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MAIL STOP PROVISIONAL APPLICATION

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c)

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TITLE OF THE INVENTION (500 characters max)

METHOD FOR ANALYZING N-TERMINAL PROTEIN ADDUCTS

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Respectfully submitted,

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PROVISIONAL APPLICATION FILING ONLY

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Method for analyzing N-terminal protein adducts

This invention concerns the technical field of analyzing adducts. In particular the present invention relates to a fluorescent/ionizable N-*R*-Edman procedure for analysis of N-terminal protein adducts with spectrophotometric and/or mass spectrometric detection methods. Further the invention relates to products necessary in the above mentioned method and uses of said products and said method.

Background

It has been demonstrated earlier that *in vivo* electrophilic compounds can be monitored by measuring the products (adducts) of their reaction with proteins, in particular hemoglobin (Hb) (*1-5*). Important nucleophilic sites in proteins, e.g., hemoglobin (Hb), which are reactive under physiological conditions are the imidazole nitrogen atoms in histidine residues, sulfur atoms in cysteine and methionine residues, oxygen atoms in carboxyl groups and in hydroxyl groups in tyrosine and serine residues, and the α-nitrogen atoms in the N-terminal valine residue of all four chains of human Hb (*6*).

The so called N-alkyl Edman procedure was developed for measurements of adducts (mainly low molecular weight adducts) to N-terminal valine residues in Hb (*7*). This method was based on the original Edman degradation procedure (*8,9*) used for protein sequencing. It was observed that N-terminal valine N-alkylated with a radioactively labeled 2-hydroxyethyl moiety from ethylene oxide was released spontaneously as a phenylthiohydantoin (PTH) under the conditions (pH >7) employed for the coupling reaction between phenyl isothiocyanate (PITC) and protein. The released PTH could be separated from unmodified N-terminal valine residues, as well as from the rest of the protein by means of extraction.

This observation led to the development of the N-alkyl Edman procedure for gas chromatographic (GC) mass spectrometric (MS) determination of Hb adducts (*10*). Because of its usefulness, the N-alkyl Edman method has been applied in a number of laboratories for research purposes, dose monitoring and hygienic surveillance (*11-16*).

A brief description of the N-alkyl Edman procedure is presented in Figure 1. A sample of the globin (isolated from red blood cells by acid precipitation) is dissolved in formamide. Pentafluorophenyl isothiocyanate (PFPITC) is then added,

together with a small amount of aqueous 1 M NaOH in order to obtain a near neutral solution. The mixture is maintained at room temperature overnight after which the temperature is raised to 45 °C for approximately two hours (*17*). The pentafluorophenylthiohydantoin (PFPTH) derivative of the terminal N-alkylvaline residues are released in high yield by this procedure and can subsequently be isolated by extraction (liquid-liquid).

Although the N-alkyl Edman procedure has become an established method for analysis of N-substituted hemoglobin adducts, the method has its restrictions, e.g., the range of adducts that can be analyzed is limited. Small adducts, e.g., ethylene oxide and propylene oxide can be quantified at pmol/g globin level, which is sensitive enough for measurement of background adduct levels (levels without known exposure). However, adducts with a few polar groups are more difficult to analyse, due to the limitations set by the GC separation system prior to the MS detection. Some of these limitations can be solved, e.g., by further derivatisation of the polar groups (*18*) . However, this approach is both time consuming and demand new procedures to be developed for each specific adduct: Furthermore, adducts with high molecular weigh (>500 mass units, mu) and/or thermo labile adducts are extremely difficult to analyze using the GC-MS based N-alkyl Edman procedure.

In order to overcome one or more of these problems and to optimize the sensitivity and increase the applicability range the "fluorescent/ionizable N-*R*-Edman procedure" (FIRE procedure), i.e. the present method, is provided. This invention is based on the principles of the original N-alkyl Edman procedure; adducted N-terminals can be detached with high selectivity compared to unmodified (normal) N-terminals and measured as their corresponding thiohydantoin derivatives after coupling with isothiocyanate Edman reagents.

Summary of the invention

The present invention solves one or more of the above problems by providing according to a first aspect a method for analyzing adducts in a fluid or a solid material suspected to contain said adducts comprising the following steps:

a) bringing said fluid and/or solid material in direct contact with a isothiocyanate reagent comprising a fluorescent and/or ionizable moiety or a reagent were the isothiocyanate group is directly bounded to an

aromatic ring or aromatic ring system, not being unsubstituted phenyl or pentafluorophenyl.

b) allowing said compound to react with adducted N-terminal protein/peptide present in said fluid and/or solid material,

c) separating the formed analytes from the reaction mixture, and

d) detecting the analytes.

The present invention also provides according to a second aspect a method for manufacturing a standard material for use in the method according to the first aspect of the present invention comprising the following steps:

a) reacting an N-adducted amino acid or adducted N-terminal terminal peptide/protein with a isothiocyanate compound comprising a fluorescent and/or ionizable molety and

b) purifying the formed thiohydantoin analyte, e.g., by separating the analyte from the reaction mixture.

The present invention also provides according to a third aspect a standard material obtainable by the method according to the second aspect of the present invention. The present invention also provides according to a fourth aspect use of a standard material according to the third aspect in a method according to the first aspect of the present invention. The present invention also provides according to a fifth aspect a container for use when analyzing adducts in a fluid or a solid material suspected for containing said adducted compounds, wherein said container comprises means for performing steps a) – c) as set out in the first aspect above. The present invention also provides according to a sixth aspect use of a method according to the first aspect for analyzing hazardous substances, such as acrylamide, ethylene oxide, and in vivo formed epoxides, e.g., glycidamide from acrylamide and styrene oxide from styrene. The present invention also provides according to a seventh aspect a kit comprising standard material according to the third aspect.

Accordingly hereby a novel method is introduced for analysis of electrophilic compounds measured *in vivo* as their corresponding adducts by means of the so-called "Fluorescent/ionizable N-*R*-Edman procedure" (FIRE procedure). The principle of the procedure is presented in **Figure 2**. This invention is based on the original observation that N-alkylated N-terminal protein adducts are detached with high selectivity from adducted proteins as their corresponding N-alkyl-valine phenylthiohydantoines after derivatisation with phenyl isothiocyanate (PITC) or

pentafluorophenyl isothiocyanate (PFPITC) in the so called N-alkyl Edman procedure. In the FIRE procedure, fluorescent and/or ionizable Edman reagents are preferably used (e.g., the isothiocyanates; FITC, DNITC and DABITC), which after reaction with adducted N-terminals in proteins are detached and analyzed as their corresponding thiohydantoines. In contrast to the N-alkyl Edman method, the FIRE procedure is designed to yield analytes which have physical and chemical properties suitable for isolation, separation and analysis in liquid based systems.

The FIRE procedure utilizes isothiocyanate reagents with ionizable functional groups, e.g., carboxyl groups and tertiary amines, in order to:

a) simplify the clean up, e.g., by enrichment of the analytes with use of ion-exchangers.
b) be able to utilize highly effective separation techniques such as electrophoresis, e.g., capillary electrophoresis (CE) and liquid chromatography (LC).
c) utilize the functional group for altering solubility's of reagents/analytes by changing the pH above or below its pK_a. For example, the carboxyl group in FITC gives the reagent (and formed analytes) increased water solubility at the pH for the coupling reaction (see **Figure 3**) as well as excellent chromatographic properties when separated on LC systems with buffered mobile phases two to three pH units below the analytes pK_a.
d) provide high sensitivities and low detection limits (LOD) when using mass spectrometric (MS) detection techniques, e.g., LC-MS, due to more effective ionization of the analytes.

The FIRE procedure utilizes isothiocyanate reagents that form analytes, thiohydantoines, which are fluorescent; this makes it possible to utilize a wider range of analytical separation and detection techniques e.g.:

a) Capillary electrophoresis with laser induced fluorescence detection (CE-LIF). This approach will give the benefit of a very high sensitivity and moderate to high selectivity. In accordance to Irland *et.al.* (*19*) who separated 18 of 20 coded FTH-amino acids, formed according to the original Edman method (*8*), analyzed with a LOD of about 10 zmol (10^{-21}).
b) LC separation using laser induced fluorescence detection (LC-LIF).

c) CE or LC with fluorescence detection. These systems are very common, easy to operate, provide good sensitivity and are relatively inexpensive.

d) CE or LC with UV detection, e.g., CE with diode array UV detector (DAD). These types of systems provide some selectivity, good reproducibility, fair sensitivity and are easy to operate.

e) LC separation using fluorescence detection combined with MS detection. This approach will give the benefits of both these techniques; both possessing high sensitivity and specificity. This will be a powerful tool for identification and determination of previously unknown adducts/ low levels of adducts (near LOD).

f) Gel electrophoresis with fluorescence detection. This approach will give the benefit of high sensitivity and possibility to run samples in parallel with possibility for repeated scans for increased sensitivity.

g) Capillary electrophoresis with application of analytes on rotating media followed by fluorescence detection. This approach will give the benefit of high separation with possibility for repeated scans for increased sensitivity.

Besides the above mentioned advantages for the different instrumental combinations, these techniques show good linearity of quantification, the FIRE procedure provides thereby new possibilities for quantification in comparison with the GC optimized N-alkyl Edman procedure

The mild and non-discriminating conditions utilized in the FIRE procedure increase the applicability range compared to now existing methods for measurements of N-terminal protein adducts. This new method should have the potency for routine analysis for hygienic surveillance, medical purpose, animal studies and for analysis of controlled substances that forms electrophillically reactive metabolites *in vivo* .

Detailed description of the invention

It is intended throughout the present description that the expression "adducted N-terminal" embraces any kind of covalently; alkylated, arylated or by other means modified N-terminal protein, but preferably N-terminals in hemoglobin, serum albumin and myoglobin. It is intended throughout the present description that the expression "LC" embraces any kind of liquid chromatography, but preferably it is

HPLC i.e. High Performance Liquid Chromatography. It is intended that the expression "MS" embraces any kind of mass-spectrometry, but preferably LC-MS.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein said adduct has been formed involving a secondary N-terminal valine.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein said adduct is a globin adduct.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein said adduct is a hemoglobin adduct.

According to a preferred embodiment of the first aspect of the invention there is provided a method, wherein said compound comprises an N=C=S-group (an isothiocyanate-group).

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein said compound is a fluorescent compound or a compound that forms fluorescent thiohydantoin analytes.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein said compound is a ionizable compound that forms ionizable thiohydantoin analytes.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein said compound is a fluorescein compound, or a derivative thereof.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein said compound is selected from the group FITC, DNITC and DABITC or a derivative thereof.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein said compound is selected from the group; 4-Isothiocyanato-benzoic acid, 4-Isothiocyanato-naphthalene-1-carboxylic acid, 10-Isothiocyanato-anthracene-9-carboxylic acid, (4-Isothiocyanato-phenyl)-dimethyl-amine, 9-Isothiocyanato-acridine, 4-Isothiocyanato-quinoline, malachite green isothiocyanate and derivative thereof.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein said compound is selected from any reagent were the isothiocyanate group is directly bounded to an aromatic ring or ring system, not being unsubstituted phenyl or pentafluorophenyl.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein said compound is FITC or a derivative thereof. The FITC may be 5' or 6' (isomer I and II).

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein detecting the reacted compound of step d) is performed at a pH above 5, preferably at a pH of about 7.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein step c) is performed by using size discriminating ultra filtration, preferably followed by an ion exchanging step, or ultracentrifugation, preferably followed by an ion exchanging step. Instead of ultracentrifugation or size discriminating ultra filtration it would be plausible to use osmotic principles to separate the un-reacted from the reacted compounds. When FITC or a derivative thereof is used in said method, an anion exchanger is preferably used in the ion exchanging step and when DNITC or DABITC or a derivative thereof is used a cation exchanger is preferably used in the ion exchanging step.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein step c) is performed by using LC.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein step c) is performed by using ion-exchange chromatography.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein step d) is performed either by using

i) LC or capillary electrophoresis and thereupon illuminating the reacted compound present and measuring the emitted energy or the absorbed energy,

ii) by using MS, preferably preceded by LC, or

iii) a combination of both i) and ii), whereby preferably i) is performed before ii).

Preferably a standard material according to the second aspect of the invention is run first in the above method whereby i), ii) or a combination of both is used, which enables that a first scan can show where the searched adduct would appear if present in the sample By using this standard material first, it will provide information of were the analyte, the adducted thiohydantoin derivative, appear and how the analyte behaves during the detection step.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein the illuminating of the reacted compound present and measuring the emitted energy or the absorbed energy is performed using LIF or a diode array detector, preferably LIF.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein the excitation wavelength is 488 nm (± 20 nm) and the measurement of the emission is performed at longer wavelengths, e.g., 515 nm.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein LC or the capillary electrophoresis in i) is followed by transferring the reacted compound present on to a rotary means, preferably a disc, and then illuminating the reacted compound present and measuring the emitted energy or the absorbed energy, whereby the illuminating of the reacted compound present and measuring the emitted energy or the absorbed energy thereof may be performed an unlimited number of times.

According to a preferred embodiment of the second aspect of the invention there is provided a method wherein said adduct is a globin adduct.

According to a preferred embodiment of the second aspect of the invention there is provided a method wherein said adduct is a hemoglobin adduct.

According to a preferred embodiment of the second aspect of the invention there is provided a method wherein said adduct is a serum albumin adduct

According to a preferred embodiment of the second aspect of the invention there is provided a method wherein said adduct is a myoglobin adduct

According to a preferred embodiment of the second aspect of the invention there is provided a method wherein said compound comprises an N=C=S-group (an isothiocyanate-group).

According to a preferred embodiment of the second aspect of the invention there is provided a method wherein said compound is a fluorescent compound, or a compound that forms fluorescent thiohydantoin analytes.

According to a preferred embodiment of the second aspect of the invention there is provided a method wherein said compound is a ionizable compound that forms ionizable thiohydantoin analytes.

According to a preferred embodiment of the second aspect of the invention there is provided a method wherein said compound is a fluorescein compound or a derivative thereof.

According to a preferred embodiment of the second aspect of the invention there is provided a method wherein said compound is selected from the group FITC, DNITC and DABITC or a derivative thereof.

According to a preferred embodiment of the second aspect of the invention there is provided a method wherein said compound is selected from the group; 4-Isothiocyanato-benzoic acid, 4-Isothiocyanato-naphthalene-1-carboxylic acid, 10-Isothiocyanato-anthracene-9-carboxylic acid, (4-Isothiocyanato-phenyl)-dimethyl-amine, 9-Isothiocyanato-acridine, 4-Isothiocyanato-quinoline, malachite green isothiocyanate and derivative thereof.

According to a preferred embodiment of the second aspect of the invention there is provided a method wherein said compound is selected from any reagent were the isothiocyanate group is directly bounded to an aromatic ring or ring system, not being unsubstituted phenyl or pentafluorophenyl (see **Figure 4**).

According to a preferred embodiment of the second aspect of the invention there is provided a method wherein said compound is FITC or a derivative thereof.

Preferred features of each aspect of the invention are as for each of the other aspects mutatis mutandis. The prior art documents mentioned herein are incorporated to the fullest extent permitted by law. The invention is further described in the following examples in conjunction with the appended figures, which do not limit the scope of the invention in any way. Embodiments of the present invention are described in more detail with the aid of examples of embodiments and figures, the only purpose of which is to illustrate the invention and are in no way intended to limit its extent.

Short description of the figures

Figure 1 shows a brief description of the N-alkyl Edman procedure.

Figure 2 shows the principles of the FI*R*E procedure, i.e. the present method.

Figure 3 shows the studied reactions and the studied reagents: Three selected fluorescent/ionizable isothiocyanate reagents; fluorescein isothiocyanate (FITC), 4-N,N-dimethylaminoazobenzene 4'-isothiocyanate (DABITC) and 4-dimethylamino-1-naphthyl isothiocyanate (DNITC) used in the FI*R*E procedure was compared with the two reagents, phenyl isothiocyanate (PITC) and pentafluorophenyl isothiocyanate (PFPITC), used in the N-alkyl Edman procedure.

Figure 4 shows general structures of analytes and proposed analytes formed with the FIRE procedure from adducted valine or asparagine as amino acids or N-terminals in peptides/proteins.

Figure 5 shows structures of compounds **1-15** as set out in the example part of the present description.

Figure 6 shows obtained by measurements of limit of detection detection LOD on LC-MS/MS in ESI and in APCI mode LC-MS/MS, positive ions, different pH, data presented on a log scale.

Figure 7 shows obtained by measurements of limit of detection detection LOD on LC-MS/MS in ESI and in APCI mode LC-MS/MS, negative ions, different pH, data presented on a log scale.

Figure 8 shows a complete baseline separation obtained with CE.

Figure 9-13 shows results form fluorescence measurements.

Figure 14-15 shows UV absorbance for selected analytes, fluoranthene used as reference

Figure 16 shows LC-MS/MS analysis of acrylamide adducted human globin by use of the FIRE procedure and comparison with reference compounds.

Figure 17 shows excitation and emission spectra of FTH-MeVal (**10**).

Examples

The FIRE procedure have been studied and evaluated utilizing three isothiocyanate reagents; fluorescein-5'-isothiocyanate (FITC), 4'-N,N-dimethylaminoazobenzene-4-isothiocyanate (DABITC) and 4-dimethylamino-1-isothiocyanate (DNITC), that all forms fluorescent thiohydantoines directly with N-substituted valines, valylpeptides (N-methylated) and proteins (alkylated globin, a few alkylating agents tested). These analytes were then compared with the reagents employed in the GC-based N-alkyl Edman procedure, PITC and pentafluorophenyl isothiocyanate (PFPITC). In this context, PITC has recently been applied for LC-MS/MS analysis of acrylamide and glycidamide adducted hemoglobin (*20*). For comparison between alkylated and non alkylated analytes, normal valine and valyl peptides were reacted with the selected isothiocyanate reagents. However, in order to form thiohydantoines from unsubstituted valine/valyl peptides an additional acidification step must be performed in accordance with the principles of the normal Edman degradation reaction (*21*).The

studied reactions and reagents are summarized in **Figure 3**, the synthesized analytes are presented with structures and abbreviations in **Figure 5**.
Selected methylated and non-methylated analytes, **(1,2; 5,6; 7,8 and 9,10)** were then compared at various pH on various analytical techniques; ultraviolet-visible spectroscopy UV/vis, HPLC, LC-MS and LC-MS/MS. The fluorescent analytes were also analysed with fluorescence spectroscopy (excitation and emission spectra) and on CE with diode array detection. It was observed that one of the fluorescent reagents was particularly well suited for analysis with these techniques.

Thus, FITC was selected for further studies using alkylated model peptides and globine adducted with acrylamide **(11)**, glycidamide **(12)**, 2-octadecyl-oxirane **(13)**, propylene oxide **(14)**, cholesterol-5α,6α-epoxide, **(15)**. These analytes **(11-15, Figure 5)** were analysed on LC-MS/MS (ESI) and were shown to give good response in the applied system. These analytes **(11-15)**, formed from in vitro alkylated globin after coupling/ detachment with FITC, were isolated by size discriminating ultra filtration (MWCO 5000) followed by anion exchanger.. The isolated extracts were then directly analyzed on LC-MS/MS successfully with a high sensitivity. Data that show the sensitivity, measured as limit of detection (LOD) for the tested reagents and the corresponding methylated and not methylated thiohydantoines are presented in **Table 1, Figure 6-7**. Results of these studies, e.g. that FTH-MeVal **(10)** give between 600 to 16.000 times higher molar sensitivity as compared to PTH-MeVal **(1)**. It was found that all selected model reagents for the FIRE procedure, FITC, DABITC and DNITC, reacts and detaches the adduct moieties with high selectively. They gave consequentially much lower LODs compared for the reagents utilized in the N-alkyl Edman procedure, down to 0.57 fmol (10^{-15}) for fluorescein N-methylvaline thiohydantoin (FTH-MeVal, **10, Table 1**) in the applied LC-MS/MS methods for analysis. This high sensitivity, measured as lower LODs, is probably a result of the incorporated ionizable groups in the reagents, which are ionized to a higher degree in MS instrument compared to adducted PTH and PFPTH analytes without ionizable groups.

In order to evaluate the possibility to measure N-terminal adducts with such sensitive techniques as CE-LIF, the fluorescent analytes, **5-10**, were also measured with fluorescence spectroscopy (excitation and emission spectra) and analyzed on CE with diode array detector (UV). The separation between FTH-Val **(9)**

and FTH-MeVal (**10**) on CE were evaluated. A complete baseline separation was obtained (**Figure 8**).

The studies on relative fluorescence were performed on the fluorescent analytes (compound **5-10**) by measurements of excitation and emission spectra. The results from this study are presented in **Figure 9-13**. The results show that FTH-MeVal (**10**) gave detectable response at the lowest concentration. FTH-MeVal (**10**) was detected at a concentration 360 times lower compared to Fluoranthene, which was used as a reference. In comparison to the other analytes FTH-MeVal (**10**) was measured at concentrations 45 times lower than DABTH-MeVal (**6**) and 530 times lower than DNTH-MeVal (**8**). The excitation and emission spectra were measured at wavelengths and pH suited for each compound.

Experimental

Material and Methods

Structures of compounds **1-15** are given in **Figure 5**.Fluoresceine isothiocyanate (isomer I, <90%), pentafluorophenyl isothiocyanate (PFPITC) and phenyl isothiocyanate (PITC, purum) were obtained from Fluka. 4-N,N-Dimethylaminoazobenzene 4'-isothiocyanate (DABITC) and 4-dimethylamino-1-naphthyl isothiocyanate (DNITC) were obtained from Acros. Cholesterole-5α,6α-epoxide, horse skeletal myoglobin, octadecyl-epoxide, L-Valine (Val), *N*-methyl-D,L-valine (MeVal) and 5-isopropyl-3-phenyl-2-thiohydantoin (**1**, Val-PTH) were obtained from Sigma. *N*-Methylvalylleucylanilide (MeValLeu-NHφ > 99 %) and valylleucylserine (ValLeuSer (H-Val-Leu-Ser-OH) 95%) were obtained from Bachem (Bubendorf, Switzerland). ($^2H_3$)Acetonitrile (99.8 % ^2H), (^2H)chloroform (99.8 % ^2H), deuterium oxide (99.9 % ^2H, ^2H_2O), and ($^2H_4$)methanol (99.8 % ^2H) were obtained from CIL (Andover, MA). 5-Isopropyl-1-methyl-3-phenyl-2-thiohydantoin (**2**, PTH-MeVal), 5-isopropyl-3-pentafluorophenyl-2-valinethiohydantoin (**3**, PFPTH-MeVal) and 1-hydroxyethyl-5-isopropyl-3-pentafluorophenyl-2-thiohydantoin (**4**, PFPTH-HOEtVal) were synthesized as described earlier (22). Glycidamide (GA) was synthesized from acrylonitrile according to Payne and Williams (23) method B. All other chemicals and solvents were of analytical grade.

The size discriminating ultra filtration tubes "Vivaspin 6" was obtained from Sartorius AG (Hannover, Germany). The ion exchanger, Amberlyst A-26, was obtained from BHD Chemicals Ltd (Poole, England)

Instrumentation, methods for analysis and characterization.
^{1}H and ^{13}C NMR spectra were recorded on a JEOL GSX 270 instrument at 270 MHz. All solvents used were fully deuterated; TMS was added as internal standard in chloroform, acetonitrile and methanol.

Absorbance spectra of the investigated reagents were obtained in water:acetonitril solutions (1:1) with no buffer added respectively 0,1% TFA, using a double-beam Hitachi U3000 UV spectrophotometer, scanning from 200 to 600 nm. A quarts cell (10 mm) was used.

Fluorescence spectra, excitation and emission, were recorded in water:acetonitril solutions (2:1) at various pH (between pH1 and pH9) using a Shimadzu spectro fluoro photometer RF5000 fluorescence spectrophotometer. Emission was scanned with the excitation wavelength locked at the optimal absorbance wavelength found for the UV absorbance measurements described above. Emission was scanned from +10nm over the locked excitation wavelength to 600nm. Excitation was scanned with the emission wavelength locked at optimum obtained during the emission wavelength scan. Excitation was scanned from 200nm to –10nm below the locked emission wavelength. Emission and excitation spectra for the analytes were compared with the same instrumental settings (slit 5) and pH suited for each analyte. A quarts cell (10 mm) was used.

TLC was performed using silica gel 60 f-254 plates (SiO_2, Merck), spots were developed with UV (254 nm) and at long wave (378 nm). Melting points were determined on a Büchi 535 instrument. Measurements of pH were carried out on an Orion EA 920 pH-meter equipped with a Ross 8130 glass electrode.

Studies of retention times of compounds **5-10** were performed using a Shimadzu LC-4A (Kyoto, Japan) HPLC with a Shimadzu SPD-2AS UV detector (λ = 268 nm, D_2 lamp). The separation column was a Kromasil LC-18 column (250 x 10 mm) with a

flow rate of 2.5 mL/min, a sample loop of 0.7 mL. The mobile phase consisted of 2 % aqueous acetonitrile buffered with 0.02 % TFA

The LC-MS system comprised a Rheos 4000LC pump (Flux Instruments, Basel, Switzerland) interfaced with a LCQ MS (ThermoQuest, CA, USA). Well adopted buffer systems, e.g., 0.1 % TFA, 0.1 % ammonium acetate and 0.3 mM aqueous ammonia, were used. For the LOD determination the flow rate was 200 µL/min of the aqueous buffer/acetonitrile [1:1 (v/v)] the analytes were dissolved in the same buffers and solvent mixtures at concentrations ranging from 10 µg/mL down to 1 ng/mL depending on their response and the operating conditions. The injection volume was 5 µL (n=3 for each compound). Nitrogen was used as drying gas at a flow rate of 250 L/h. Both the positive- and the negative-ion mode were used. Determinations in MS-MS mode was performed by collision-induced dissociation (CID) of the [M+1] ion. When the MS was operated in electrospray ionization (ESI) mode. The mobile phase consisted of 1:1, H_2O:acetonitrile at an isocratic flow at 200 µL/min. The ion-source temperature was 120 °C, capillary temp 250 °C, capillary voltage 10 V, nitrogen was used as sheath gas and the cone voltage varied between 25 and 140 V order to obtain maximum sensitivity for the each specific analyte. When operated in atmospheric pressure chemical ionization (APCI) following settings were applied: The mobile phase consisted of 1:1, H_2O:acetonitrile at an isocratic flow at 500 µL/min. The vaporation temperature was 450 °C, nitrogen was used as sheath gas, capillary temp 150 °C and a capillary voltage of 5 V.

The CE separation was performed on an HP 3D CE (Agilent, CA, USA) with a five channel diode array UV detector. A fused silica capillary (i.d. 50 µm, o.d. 375 µm) with a total length of 64 cm and an effective length of 56 cm was used. The separation voltage was +30 kV, resulting in a separation current of 32 µA. The buffer system consisted of a 17 mM phosphate buffer (adjusted to pH 7) containing 20 mM SDS and the injection volume was 1 nL.

Synthesis of reference compounds/analytes

Synthesis of 4-N,N-Dimethylaminoazobenzene 4'-thiohydntion-valine (5, DABTH-Val).

From a stock solution of 0,500 M L-valine (20mmol in 40ml 0.25 M KOH), an aliquot 5.0 mL (2.5 mmol MeVal) was heated to 60°C and mixed with 5.0 ml 0.10 M 4-N,N-Dimethylaminoazobenzene 4'-isothiocyanate (DABITC, 0.5 mmol) in dioxan. In order to achieve a homogeneous solution another 3.5 mL dioxan and 2.5 mL aq were added. The reaction was monitored on TLC (EtOAc/MeOH 4:1) and after 30 min the reagent, DABITC, was consumed. Concentrated HCl (1mL, 12 mM) was added in order to convert the formed 4-N,N-Dimethylaminoazobenzene 4'-thiocarbamoyl-valine to the correspondingly ring closed DABTH-Val (5).The reaction was monitored on TLC (Tol:EtOAc 2:1, spots developed with UV and long wave) and was found to be completed after 30 min at 60°C. The solution was extracted from chloroform (25mL) and water (25mL). The isolated water phase was neutralized with $KHCO_3$, and the product extracted with chloroform (2x 25mL). The chloroform phase was extracted with water (25mL) and the solution was dried with Na_2SO_4. After filtration and evaporation the obtained solid (orange color), was crystallized from DCM:hexan (2:1) to yield 131.7 mg (69.0 %), m.p. 225°C. Rf-TLC 0, 73(EtOAc:MeOH 4:1). UV (acn:water 1:1) λ_{max}=265 nm, ε_{265}=28000, see also **Figure 15**. ^{1}H NMR($CDCl_3$,25°C) δ1.06, 1.15[d+d, 3+3H, *J*=6.9, 7.1 Hz, $CH_3(\gamma,\gamma')$], 2.39[m, 1H, *J*=3.3, 6.9, 7.1Hz, $CH(\beta)$], 3.09 [s, 6H, $N(CH_3)_2$] 4.18 [d, 1H, *J*=3.3, $CH(\alpha)$], 6.75, 6.77 [d+d, 2H, azobenzene C_6-*H*,C_7-*H*] 7.39, 7.41 [d+d, 2H, azobenzene C_5-*H*,C_8-*H*] 7.88, 7.90, 7.94, 7.96 [4d, 4H, azobenzene C_1-*H*-C_4-*H*].

Synthesis of 4-N,N-dimethylaminoazobenzene 4'-tiohydantion-methylvaline (6, DABTH-MeVal). From a stock solution of 0,500 M *N*-(Me)-D,L-valine (20mmol in 40mL 0,25 M KOH), an aliquot 5.0 mL (2,5mmol MeVal) were heated to 60°C and mixed with 0,100M 4-N,N-Dimethylaminoazobenzene 4'-isothiocyanate (DABITC) in dioxan (5.0mL, 0.5 mmol). In order to achieve a homogeneous solution another 3.5 mL dioxan and 2.5 mL aq were added. The MeVal reaction mixture became inhomogeneous in comparison to the Val solution, indicating that the formed DABTH-MeVal precipitated. The reaction was monitored on TLC (SiO_2, Tol/EtOAc 2:1,spots developed with UV and long wave), after 30 min all the DABITC were consumed. The extraction was performed as described above. The product crystallized from DCM:hex (1:4, v/v) to yield 189.0mg (99.5 %), m.p.164.5-169°C, Rf-TLC 0.73 (SiO_2,

EtOAc:MeOH 4:1). UV (acn:water 1:1) λ_{max}=265 nm, ε_{285}=26000, see also **Figure 14**, fluorescence measurements (see **Figure 11, 12**). ^{1}H NMR($CDCl_3$,25°C) δ1.05, 1.25[d+d, 3+3H, *J*=6.9, 7.1Hz, C*H*$_3$(γ,γ')], 2.47[m, 1H, *J*=3.3, 6.9, 7.1Hz, C*H*(β)], 3.09 [s, 6H, N(C*H*$_3$)$_2$] 3.4[s, 3H, N-C*H*$_3$], 4.4 [d, 1H, *J*=3.3Hz, C*H*(α)], 6.74, 6.77 [d+d, 2H, azobenzene C_6-*H*,C_7-*H*] 7.37, 7.40 [d+d, 2H, azobenzene C_5-*H*,C_8-*H*] 7.87, 7.90, 7.92, 7.95 [4d, 4H, azobenzene C_1-*H*-C_4-*H*].

Synthesis of 4-Me$_2$N-naftyl)valine-thiohydantoin (7, DNTH-Val). L-Valine (Sigma, 804 mg, 6.86 mmol) was alkalized with KOH (3.43 mmol) and dissolved in 0.5 M $KHCO_3$ (3 mL) and dioxan (3 mL). The solution was heated to 45 °C with magnetic stirring and DMNaf-ITC (88 mg, 0.385 mmol) dissolved in dioxan (2 mL) was added to the suspended solution. The reaction was monitored on TLC (SiO_2, Ethyl acetate / MeOH 4:1, toluene and 2:1 toluene / ethyl acetate) with a reference without addition of valine. After 60 min the reaction was in principal completed, no DNITC could be detected on TLC and the formed product, the 4-Me_2N-Naftyl)-thiocarbamoyl-valinate gave fluorescence at long wave under the UV-lamp (tailing spot on TLC eluted with Ethyl acetate / MeOH 4:1). The reaction was acidified with 3 mL konc hydrochloric acid (36 mmol) and the reaction was stopped after 2 h, at 45 ° by neutralization with solid $KHCO_3$ until no more carbon dioxide was released. The reaction mixture was diluted with water (100 mL) and the product was purified by extraction with $CHCl_3$ (125 ml). The organic phase was dried with Na_2SO_4, filtered and evaporated to yield 126 mg, yield 0.96 % as a white solid m.p. 107,5-110°C. Rf-TLC 0. 73 (EtOAc:MeOH 4:1). UV (acn:water 1:1) λ_{max}=260 nm, ε_{260}=21000, see also **Figure 14, 15**. ^{1}H NMR($^2H_6$-aceton, 25°C) δ1.10, 1.25[d+d, 3+3H, *J*=6.9, 7.1Hz, C*H*$_3$(γ,γ') assigned to the thiohydantoin], 1.17-1.21[d+d, 3+3H, *J*=6.9 Hz, C*H*$_3$(γ,γ') assigned to the thiocarbamoyl], 2.40[m, 1H, *J*=3.8, 6.9Hz, C*H*(β)], 2.92 [s, 6H, N(C*H*$_3$)$_2$] 4.45, 4.56 [d+d, 1H, *J*=1.65, 3.85Hz, C*H*(α)], 7.17-8.32 [m, 6H, naftalene-*H*]. ^{13}C NMR (acetone, 25°C) δ 16.8, 17.4; 18.7, 19.0 [2x C*H*$_3$(γ,γ')], 31.66; 32.0 [2x C*H*(β)], 45.4 [N-(C*H*$_3$)$_2$], 65.7; 66.1 [2x C*H*(α)], 114-153 [naftalen], 174.9 [CO], 185.8; 185.9 [2x CS]. This molecule was found to be partly hydrolyzed to the corresponding ring opened thiocarbamoyl compound. All obtained shifts are presented but not assigned to the respective compound.

*Synthesis of N-Me(4-Me$_2$N-Naftyl)valine-tiohydantoin (*8,.DNTH-MeVal*).* D,L-MeVal (prepared according to Rydberg *et al* 93, 93.0 mg, 0.708 mmol) was alkalized with KOH (0.4 mmol) and dissolved in 0.5 M $KHCO_3$ (3 mL) and dioxan (2 mL). The solution was heated to 45 °C with magnetic stirring and p-dimethylnafthylisothiocyanate (DNITC, 91.3 mg, 0.40 mmol) dissolved in dioxan (2 mL) was added and the solution. The reaction was monitored on TLC (SiO_2, toluene and 2:1 toluene / ethyl acetate) with a blank reference (DNITC dissolved in the solvent mixture). After 60 min the reagent, DNITC, was consumed and the formed analyte could be developed at on first TLC eluted with toluene/ethyl acetate (with fluorescence at long wave under the UV-lamp). The reaction was extracted with toluene and purified by column chromatography eluted with toluene/ethyl acetate (SiO_2, 2/1) to yield 130 mg, 96 % yield. The product was crystallized from ethanol/water (1:1), to yield white crystals (100 mg, 73 %), m.p. 157-159°C, Rf-TLC 0.72(EtOAc:MeOH 4:1), UV (acn:water 1:1) λ_{max}=260 nm, ε_{260}=21000, see also **Figure 14, 15.** fluorescence measurements (see **Figure 12).** ^{1}H NMR($^2H_6$-aceton,25°C) δ1.04, 1.16[d+d, 3+3H, *J*=6.9, 7.1Hz, C*H*$_3$(γ,γ´)assigned to the thiohydantoin], 1.24, 1.28[d+d, 3+3H, *J*=6.9 Hz, C*H*$_3$(γ,γ´) assigned to the thiocarbamoyl], 2.59[m, 1H, *J*=3.3, 6.9, 7.1Hz, C*H*(β)], 2.92 [s, 6H, N(C*H*$_3$)$_2$] 3.41, [d, 3H, *J*=1.65, Hz, N-C*H*$_3$] 4.38, 4.50 [d+d, 1H, *J*=3.0, 3.3Hz, C*H*(α)], 7,15-8,31 [m, 6H, naftalene-*H*]. ^{13}C NMR (acetone, 25°C) δ 16.3, 17.1; 17.5, 17.8 [2x C*H*$_3$(γ,γ´)], 30.3 [*C*H(β)], 33.0; 33.1 [2x N-*C*H$_3$], 45.4 [N-(*C*H$_3$)$_2$], δ69.1; 69.4 [2x *C*H(α)], 114-153 [2x naphtalene], 173.5 [CO], 184.3; 184.4 [2x CS]. This molecule was found to be partly hydrolyzed to the corresponding ring opened thiocarbamoyl compound. All obtained shifts are presented but not assigned to the respective compound.

Synthesis of Fluoresceintiohydantoin-valin (9, FTH-Val). From a stock solution of 0,500 M L-valine (20mmol in 40ml 0,25 M KOH), an aliquot, 5.0 mL (2,5mmol L-valine) was heated to 45°C and then reacted with FITC (0.5 mmol, 199.2 mg) disolved in 6 mL dioxane/water (10:1). The reaction was followed on TLC and after

90 min all the FITC was consumed. Konc HCl (1mL, 12 mM) was added in order to convert the formed 5´-fluorescein thiocarbamoyl-valininate to the correspondingly cyclized FTH-Val, after 14 h at 45°C the reaction was completed. The product was evaporated under vacuum and the dry solid dissolved in water: EtOAc (1:1, tot 60mL). The EtOAc phase was extracted with water (15 mL x2). The combined aqueous phase was then extracted with EtOAc (20 mL) and combined with the first EtOAc-phase this was finally extracted with water (2x 15 mL). The organic phase was dried with Na_2SO_4 and the product was dissolved in EtOAc:MeOH (4:1) and purified by column chromatography (SiO_2, 25 x 3 cm) eluted with EtOAc:MeOH (4:1).The fractions containing the products were combined, evaporated and crystallized from EtOH:water (1:1) to yield 167 mg (68,4%), m.p. 232,5-234,5°C. Rf-TLC 0,65 (EtOAc:MeOH 4:1). UV (acn:water 1:1) λ_{max}=268 nm, ε_{268}=21500, see also **Figure 14, 15**. Fluorescence measurements (see **Figure 9, 12**). ^{1}H NMR(aceton,25°C) δ1.08, 1.18[d+d, 3+3H, *J*=6.9, 6.6Hz, C*H*$_3$(γ,γ´)], 2.38[m, 1H, *J*=3.8, 6.9Hz, C*H*(β)], 2.86 [s, 1H, not assigned] 4.4[d, 1H, *J*=3.8Hz, C*H*(α)], 6.66-6.77 [6H, m with the most pronounced peak at 6.71, xanthene-*H*], 7.39, 7.42 [d+d, 1H, *J*=0.55, 8.2Hz, C_7-*H*] 7.74, 7.77 [d+d, 1H, *J*=1.9, 8.2Hz, C_8-*H*], 7.96 [d, 1H, *J*=1.6Hz, C_4-*H*] 9,0 [s, 2H, xanthene-(O*H*)$_2$] 9,5 [s, 1H, N-*H*]. ^{13}C NMR (acetone, CD_3OD, 25°C) δ 17.0, 18.9 [CH_3(γ,γ´)], 32.5 [CH(β)], 66.3[CH(α)], 103.7, 113.8, 125.9, 126.1, 130.5, 154.0, 161.3 [xanthene carbons], 136.5, 136.9 [C_2+C_5], 169.9 [COO], 175.2 [CO], 184.6 [CS]. The chemical shifts of five of the carbons is not given, (impossible to separate from background noise), however, the presented carbons were in accordance to the assumed shifts of compound **9** in its assumed spiro-conformation (see **Figure 3**).

*Synthesis of 5´fluoresceintiohydantoin-N-methylvalin (**10**, FTH-MeVal)* From a stock solution of 0,500 M *N*-(Me)-D,L-valine (20mmol in 40ml 0,25 M KOH), an aliquot, 5.0 mL (2,5mmol MeVal) were heated to 45°C and then reacted with FITC (0.5 mmol, 199.2 mg) dissolved in 6 mL dioxan/water (10:1). The reaction was monitored on TLC and after 90 min all the FITC was consumed. In order to extract the formed product, FTH-MeVal were acidified with konc HCl (1mL, 12 mM) and extracted as described above. After evaporation to dryness was the product purified on column and

crystallized as described above to yield 194,4 mg (77,4%) , m.p. 213,5-217°C. Rf-TLC 0,64 (EtOAc:MeOH 4:1). UV (acn:water 1:1) λ_{max}=268 nm, ε_{268}=17500, see also **Figure 14, 15.** fluorescence measurements (see **Figure 10, 12**). ^{1}H NMR(aceton,25°C) δ1.01, 1.25[d+d, 3+3H, *J*=6.9Hz, C*H*$_3$(γ,γ')], 2.57[m, 1H, *J*=3.6, 6.9Hz, C*H*(β)], 2.87 [s, 1H, not assigned] 3.4[s, 3H, N-C*H*$_3$], 4.4[d, 1H, *J*=3.0Hz, C*H*(α)], 6.65-6.77 [6H, m with the most pronounced peak at 6.71, xanthene-*H*], 7.39, 7.42 [d+d, 1H, *J*=0.55, 8.2Hz, C$_7$-*H*] 7.72, 7.75 [d+d, 1H, *J*=1.9, 8.2Hz, C$_6$-*H*], 7.93 [d, 1H, *J*=1.9Hz, C$_4$-*H*] 9,0 [s, 2H, xanthene-(O*H*)$_2$]. ^{13}C NMR (acetone, 25°C) δ 16.3, 17.5 [C*H*$_3$(γ,γ')], 30.3 [CH(β)], 33.1 [N-C*H*$_3$], 69.3[CH(α)], 103.5, 113.5, 125.4, 125.7, 130.2, 153.4, 153.8, 160.5 [xanthene carbons]136.6 [C$_2$ or C$_5$], 168.7 [COO], 172.7 [CO], 182.7 [CS]. The chemical shifts of six of the carbons is not given, (impossible to separate from background noise), however, the presented carbons were in accordance to the assumed shifts of compound **10** in its assumed spiro-conformation (see **Figure 3**).

Synthesis of 5'fluoresceintiohydantoin-N-2-carbamoylethyl-valine (11, FTH-AAVal) Valine (117 mg, 1.0 mol) was dissolved in 10 mL water/dioxan (4:1), pH was adjusted to around 10 by addition of NaOH (20 mg, 0.50 mmol). To this solution acrylamide (71 mg, 1.0 mmol) was added and the solution was heated at 45 °C for three days. An aliquot of this mixture (2.5 mL, containing 0.25 mmol reacted valine) was directly reacted with FITC (20 mg, 0.051 mmol) at 60 °C for 90 min. The reaction mixture was acidified with 0.5 mL 1 M HCl (0.50 mmol), and the product was dissolved and extracted in water (10 mL) and EtOAc (10 mL), the EtOAc phase was extracted with another two portions of water (10 mL). The EtOAc phase was dried with Na_2SO_4, evaporated to dryness to yield FTH-AAVal (25 mg, 0,046 mmol, 90%) with small amount of impurities revealed by TLC (SiO_2 EtOAC: MeOH, 4:1). Characterization was performed on LC-MS (ESI) in both positive and negative mode.

In vitro alkylation of globine with glycidamide, octadecyl epoxide, propylene oxide and *cholesterole-5α,6α-epoxide*. General procedure; Four portions of globine (50 mg) isolated from hemoglobin according to Mowrer *et.al* (*24*) were dissolved in 2 mL 0.5

M aqueous $KHCO_3$:2-propanol (2:1) and alkylated with glycidamide (10 mg, 0.11 mmol), octadecyl epoxide (10 mg, 0.037 mmol), propylene oxide (10 mg, 0,17 mmol) and cholesterole-5α,6α-epoxide (10 mg, 0.025 mmol), respectively. Additional portions of dioxan, 1 mL each, were added in order to get the samples containing octadecyl epoxide and cholesterole-5α,6α-epoxide homogenous. The reaction mixtures were heated to 60 °C for three days, purified from the excess alkylating reagents/by products by use of size discriminating ultra filtration (samples diluted with water 2-propanol (2:1), the samples containing dioxan were evaporated first). The alkylated globines were dissolved and derivatised with FITC as described below.

FITC derivatisation of alkylated globines and analysis of formed FTH derivatives; 5'fluoresceintiohydantoin-N-2-carbamoylethyl-valine (11, FTH-AAVal), 5'fluoresceintiohydantoin-N-2-carbamoy-2-hydroxylethyl-valine (12, FTH-GAVal), 5'fluoresceintiohydantoin-N-2-hydroxyoctadecyl-valine (13, FTH-HOC$_{18}$Val), 5'fluoresceintiohydantoin-N-2-hydroxypropyl-valine (14, FTH-GAVal) and 5'fluoresceintiohydantoin-N-(5α and or 6α)-hydroxycholesterole-valine (15, FTH-CholEOVal) The above alkylated globin samples (*n*=4), akrylamid alkylated globine (50 mg, 7-10 nmol acrylamide/g globine, gift from Birgit Paulsson) including one control globin (50 mg, normal globine) and one control myoglobin (50 mg, horse skeletal myoglobin, Sigma), were separately dissolved in 2 mL 0.5 M aqueous KHCO3/ 2-propanol (2/1) and reacted with FITC (15 mg, 0.038 mmol). After 90 min at 60 °C, the reaction mixtures were diluted with 2 mL of water:2-propanol (2/1) and transferred to size discriminating ultra filtration tubes ("Vivaspin 6" from (Sartorius AG), MWCO 5000). The tubes were centrifuged about 3-4 hours at maximum speed on a standard laboratory centrifuge. The analytes were purified on anion exchangers (Amberlyst A-26, hydroxyl ion as counter ion) and eluted with 0.5 % TFA, the extract was evaporated and dissolved in 1 mL water/acetonitrile (1/1) and directly analyzed on LC-MS(ESI) with direct injection and/or column separation (C_{18}). The solutions containing compound **11-15** were characterized on LC-MS (ESI) in both positive and negative mode.

Results

To evaluate the potency of the FIRE procedure the derivatives of the selected fluorescent isothiocyanate reagents (compound **5-10, Figure 5**) were analysed and compared to the derivatives of the isothiocyanate reagents used in the N-Edman procedure (compound **1-4, Figure 5**). Selected analytes were analysed on; ^{1}H NMR (**1-10**), ^{13}C NMR (**1-5, 7-10**), HPLC-UV (**1-2, 5-6, 7-8, 9-10**), LC-MS/MS (**1-15**), CE-DAD (**9, 10**) and UV/vis spectroscopy (**1, 2, 6-10**). Compounds **5-10** were also measured on fluorescence spectroscopy. In general, for all measurement and determinations the pH was alternated below and above the pKa for the respective analyte.

NMR-Results. ^{1}H and ^{13}C NMR were used in order to characterise the synthesised compounds; compound 1-4, have been synthesized and described earlier (Rydberg 93), compound **5-10** (normally 10 mg for ^{1}H NMR and 40 mg for ^{13}C NMR) were dissolved in fully deuterated solvents. The NMR analysis gives valuable information, e.g., the well separated shifts for the γ, γ'- methyls (dd, in ^{1}H NMR) in the valine spin residue can be used to confirm that the compounds are fully ring closed thiohydantoins and not its precursors, i.e., in the correspondingly thiocarbamoylated compounds the γ, γ'- methyls are not as well separated.

Fluorescence

The studies on relative fluorescence were performed for the fluorescent analytes/compound by recording of excitation and emission spectra. The results are presented in **Figure 9-13**. The FITC derivatives; FTH-Val (**9**) and FTH-MeVal (**10**) showed the highest relative fluorescence (at pH > 5) compared to the other used reagents/compounds. FTH-MeVal (**10**) was detected at a concentration 360 times lower compared to fluoranthene (used as a reference), approximately 45 times lower than for DABTH-MeVal (**6**) and 530 times lower than DNTH-MeVal (**8**). In addition, FTH-Val and FTH-MeVal give an almost identical response, indicating that the adduct, e.g., the methyl group in FTH-MeVal (**9**) does not effect the spectroscopic properties in comparison with FTH-Val (**8**).

Liquid chromatography

Using LC, it was possible to separate the corresponding tiohydantoins, methylated and non-methylated, for all four tested reagents (1-2, 5-6, 7-8 and 9-10). Different

gradients were used for the different reagents and, in spite of the relatively small structural differences between the methylated and the non-methylated molecules, baseline separations were achieved using a standard C-18 column. A chromatogram of the LC-MS separation is presented in **Figure 16**, were the FTH-acrylamide adduct (**11**), FTH-MeVal (**10**) and FTH-Val (**9**) are separated using LC and identified with MS. The experiment was performed using acrylamide alkylated human globin and corresponding FTH-adducts **9-11** were used as references (see **Figure 16**).

Mass spectrometry

In order to evaluate the suitability/applicability of the fluorescent isothiocyanate derivatives on LC-MS/MS, compounds **1-10** were compared. The cone voltage, polarity, pH and the composition of the buffer system was varied and optimized for each analyte. The results from this study are summarized in **Figures 6-7** and in **Table 1.**

n.m = not measured

Table 1. Comparison of relative sensitivities obtained by measurements of limit of detection detection LOD on LC-MS/MS in ESI and in APCI mode, using various pH modifiers/buffers. LOD were meaured on positive and negative ions with direct injections (5 ul loop). The lowest obtained sensitivity is set to one, and the others are set relative to FTH-MeVal in 0.3 mM NH_3, negative ions measured (coloured/ dashed), this where calculated to be 0.57 fmol.

Buffer	0.1% NH4oAc	0.1% TFA	0.3mM NH3	Un-buffered	0.1% TFA	NH4oAc	0.3mM NH3	Un-buffered	NH4oAc
Ionization method measured Ions	ESI positive	ESI positive	ESI positive	ESI positive	APCI positive	ESI negative	ESI negative	ESI negative	APCI negative
FTH-MeVal	77.0	18.8	n.m	2180	1.7	67.1	[illegible]	244	294
FTH-Val	17.4	5.5	22.5	1910	3.1	7.6	n.m	2790	n.m
DABTH-MeVal	294	131	47.8	1840	20.4	220	272	1880	n.m
PTH-MeVal	48700	38100	2890	176600	3800	32200	4390	4360	n.m
DNTH-MeVal	4580	442	2240	4530	42.3	1050000	76600	4791	n.m
PFPTH-HOEt	n.m	n.m	n.m	n.m	30600	n.m	n.m	26500	1640
PFPTH-MeVal	n.m	n.m	n.m	n.m	352000	n.m	n.m	n.m	n.m

Compound **8** and **9**, FTH-Val and FTH-MeVal, gave the highest response for the tested conditions for the LC-MS. The LOD was 0.57 fmol (10^{-15}) for FTH-MeVal under

optimal conditions, which is about 600 times lower compared to the traditional N-Edman reagent adduct, PTH-MeVal. The high sensitivity for the FITC derivatives is probably a result of the incorporated ionizable moiety in the reagent, which is ionized to a higher degree in the MS ion source compared to the PTH and PFPTH derivatives. To investigate the applicability range for this reagent, valine and globine were adducted with glycidamide, propylene oxide and the high molecular weight adducts cholesterole-5α,6α-epoxide and octadecyl-epoxide. The formed thiohydantoines (compounds **11-15**) were then characterized and determined on LC-MS/MS.

Capillary electrophoresis

The separation between FTH-MeVal and FTH-Val on a CE-DAD system was evaluated to assess the possibility to determine N-terminal adducts using other separation techniques than LC and to be able to utilize and benefit from such sensitive techniques as CE-LIF. The separation was performed using the previously stated conditions e.g., 17 mM phosphate buffer (adjusted to pH 7) containing 20 mM SDS. As can be seen in **Figure 8**, a complete baseline separation was obtained, FTH-Val elutes at 7.44 min and FTH MeVal elutes at 8.91 min

All tested fluorescent isothiocyanate reagents react and detach the adduct moiety with a high selectivity. The LC-MS/MS and LC-fluorescence detection possesses a high sensitivity and selectivity and the combination of these techniques provide a powerful tool for identification of unknown compounds and for studies of adduct patterns between species and/or within species. It was observed that fluorescein isothiocyanate (FITC) was exceptional, regarding the separation, solubility, sensitivity on MS and spectroscopic properties in comparison to the other tested reagents. Thus, FITC was selected for further studies using alkylated model peptides and globine. The isolated extracts were then directly analyzed on LC-MS/MS successfully with a high sensitivity and high selectivity in the applied system.

It was possible to isolate these adducts from alkylated globin derivatized with FITC, by size discriminating ultra filtration followed by clean-up utilizing an anion exchanger. This approach is not only time saving but is also non-discriminating especially for analysis of adducts with high polarity as they often are lost or gives poor yields on liquid/liquid or solid phase extraction clean up steps.

The results presented above also show that the FITC derivatives; FTH-Val (9) and FTH-MeVal (**10**) are well suited for separation on CE (**Figure 8**) using fluorescence detection at specific excitation and emission at wavelengths around 492 and 515 nm respectively (**Figure 17**). This make the FITC derivatives suitable for excitation using an Argon-ion laser (488 nm) and opens up the possibility to utilize CE-LIF for adducts measurements down to the zmol (10^{-21}) levels. These results are truly encouraging for miniaturizing the fluorescent-N-*R* Edman procedure, aiming to measure adduct spectra from a few µL blood, easily available by a prick in the fingertip

The utilization of the 'FIRE procedure' in comparison to the 'N-alkyl Edman procedure' makes it possible to:

1) Increase the range of adducts to be analyzed, e.g.:
   a) high molecular weight adducts (>500 mu) as well as adducts with lower molecular weights can be readily analyzed using e.g. LC and CE.
   b) very polar to non polar adducts can be analyzed using e.g. LC and CE.
   c) thermo labile adducts can be analyzed using e.g. LC and CE.
2) Increase the number of possible separation techniques to be used for adducts analysis.
3) Improve and speed-up the sample clean up and extraction.
4) Improve the sensitivity for the detection of the analytes using e.g. LC-MS/MS and LIF.

Various embodiments of the present invention have been described above but a person skilled in the art realizes further minor alterations, which would fall into the scope of the present invention. The breadth and scope of the present invention should not be limited by any of the above-described exemplary embodiments, but should be defined only in accordance with the following claims and their equivalents. For example, any of the above-noted methods can be combined with other known methods. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

References

1. Ehrenberg, L., Hiesche, K. D., Osterman-Golkar, S. and Wennberg, I. (1974) Evaluation of genetic risks of alkylating agents: Tissue doses in the mouse from air contaminated with ethylene oxide. *Mutat. Res.* 24, 83-103.
2. OstermanGolkar, S., Ehrenberg, L., Segerbäck, D. and Hällström, I. (1976) Evaluation of genetic risks of alkylating agents. II. Haemoglobin as a dose monitor. *Mutat. Res.* 34, 1-10.
3. Neumann, H. G., Baur. H and Wirsing. R. (1980) Dose-response relationship in the primary lesion of strong electrophilic carcinogens. *Arch. Toxicol. Suppl.* 3, 69-77.
4. Skipper, P. L. and Tannenbaum, S. R. (1990) Protein adducts in the molecular dosimetry of chemical carcinogens. *Carcinogenesis* 11, 507-518.
5. Törnqvist, M., Fred, C., Haglund, J., Helleberg, H., Paulsson, B. and Rydberg, P. (2002) Protein adducts: quantitative and qualitative aspects of their formation, analysis and applications. *J. Chromatogr. B* 778, 279-308.
6. Osterman-Golkar S. and Ehrenberg, L. (1980) Alkylation of macromolecules for detecting mutagenic agents.*Teratog. Carcinog. Mutag.* 1, 105-127.
7. Jensen, S., Törnqvist, M. and Ehrenberg, L. (1984) Hemoglobin as a dose monitor of alkylating agents: Determination of alkylation products of N-terminal valine. (F.J.de Serres and R.W.Pero, Ed.) pp 315-320, Plenum Press, New York
8. Edman, P. (1950) Methods for determination of the amino acid sequence in peptides. *Acta Chem. Scand.* 4, 283-298.
9. Edman, P., and Henschen, A. (1975) Sequence determination. In *Protein Sequence Determination* (Needleman S. B., Ed.) pp 232-279, Springer-Verlag, Berlin
10. Törnqvist, M., Mowrer, J., Jensen, S. and Ehrenberg, L. (1986) Monitoring of environmental cancer initiators through hemoglobin adducts by a modified Edman method. *Anal. Biochem.* 154, 255-266.
11. Tates, A. D., Grummt, T., Törnqvist, M., Farmer, P. B., van Dam, F. J., van Mossel, H., Schoemaker, H. M., Osterman-Golkar, S., Uebel, C., Tang, Y. S., Zwinderman, A. H., Natarajan, A. T. and Ehrenberg, L. (1991) Biological and chemical monitoring of occupational exposure to ethylene oxide. *Mutat. Res.* 250, 483-497.
12. Törnqvist, M., Magnusson, A. L., Farmer, P. B., Tang, Y. S., Jeffery, A. M., Wazneh, L., Beulink, G. D. T., van der Waal, H. and van Sittert, N. J. (1992) Ring test for low-levels of *N*-(2-hydroxyethyl)valine in human hemoglobin. *Anal. Biochem.* 203, 357-360.
13. Bergmark, E., Calleman, C. J., He, F. and Costa, L. G. (1993) Hemoglobin adducts in humans occupationally exposed to acrylamide.*Toxicol.Appl.Pharmacol.* 120, 45-54.
14. Fidder, A., Noort, D., de Jong, A. L., Trap, H. C., de Jong, L. P. and Benschop, H. P. (1996) Monitoring of in vitro and in vivo exposure to sulfur mustard by GC/MS determination of the N-terminal valine adduct in Hb after a modified Edman degradation. *Chem. Res. Toxicol.* 9, 788-792.

15. Thier, R., Lewalter, J., Kempkes, M., Selinski, S., Bruning, T. and Bolt, H. M. (1999) Haemoglobin adducts of acrylonitrile and ethylene oxide in acrylonitrile workers, dependent of polymorphisms of the glutathione transferases GSTT1 and GSTM1. *Occup. Environ. Med.* 56, 197-202.
16. Begemann, P., Sram, R. J. and Neumann, H. G. (2001) Hemoglobin adducts of epoxybutene in workers occupationally exposed to 1,3-butadiene. *Arch. Toxicol.* 74, 680-687.
17. Törnqvist, M. (1994) Epoxide adducts to N-terminal valine of hemoglobin. In *Methods Enzymol.* (Everse J., Vandergriff K. D. and Winslow R. W., Eds.) pp 650-657, Academic Press, New York
18. Paulsson, B., Athanassiadis, I., Rydberg, P. and Törnqvist, M. (2003) Hemoglobin adducts from glycidamide: acetonization of hydrophilic groups for reproducible gas chromatography/tandem mass spectrometric analysis. *Rapid. Commun. Mass. Spectrom.* 17, 1859-1865.
19. Ireland, I. D., Lewis, D. F., Li, X., Renborg, A., Kwong, S., Chen, M. and Dovichi, N. J. (1997) Duoble Coupling Edman Chemistry for High-sensitivity Automated Protein Sequencing. *J. Protein Chem.* 16, 491-493.
20. Ospina, M., Vesper, H., Licea-Perez. H. and Luchuan, G.L. LC/MS/MS method for the analysis of acrylamide and glycidamide hemoglobin adducts. ACS, 2004
21. Edman, P. (1950) Methods for determination of the amino acid sequence in peptides. *Acta Chem. Scand.* 4, 283-298.
22. Rydberg, P., Lüning, B., Wachtmeister, C. A. and Törnqvist, M. (1993) Synthesis and characterization of *N*-substituted valines and their phenyl-thiohydantoins and pentafluorophenyl-thiohydantoins. *Acta Chem. Scand.* 47, 813-817.
23. Payne, G. B. and Williams, P. H. (2004) Reaction of hydrogen peroxide. VI. Alkaline epoxidation of acrylonitrile. *JOC.* 26, 651-659.
24. Mowrer, J., Törnqvist, T., Jensen, S. and Ehrenberg, L. (1986) Modified Edman degradation applied to haemoglobin for monitoring occupational exposure to alkylating agents. *Toxicol. Environ. Chem.* 11, 215-231.

Claims

1. Method for analyzing adducts in a fluid or a solid material suspected for containing said adducts comprising the following steps:
   a) bringing said fluid and/or solid material in direct contact with a compound comprising a fluorescent and/or an ionizing moiety.
   b) allowing said compound to react with adducts present in said fluid and/or solid material,
   c) separating the un-reacted compound from the reacted compound, and
   d) detecting the reacted compound.

2. A method according to claim 1 wherein said adduct has been formed involving a secondary N-terminal valine.

3. A method according to claim 1 wherein said adduct is a globin adduct.

4. A method according to claim 1 wherein said adduct is a hemoglobin adduct.

5. A method according to claim 1 wherein said adduct is a serum albumin adduct.

6. A method according to claim 1 wherein said compound comprises an N=C=S-group (an isothiocyanate-group) directly bounded to an aromatic ring or aromatic ring system but not to unsubstituted phenyl or pentafluorophenyl (i.e., PITC and PFPITC).

7. A method according to claim 1 wherein said compound is a fluorescein compound or a derivative thereof.

8. A method according to claim 1 wherein said compound is an ionizable compound or a derivative thereof.

9. A method according to claim 1 wherein said compound is an isothiocyanate compounds other than PITC and PFPITC.

10. A method according to claim 1 wherein said compound is selected from the group FITC, DNITC and DABITC or a derivative thereof.

11. A method according to claim 1 wherein said compound is selected from the group 4-Isothiocyanato-benzoic acid, 4-Isothiocyanato-naphthalene-1-carboxylic acid, 10-Isothiocyanato-anthracene-9-carboxylic acid, (4-Isothiocyanato-phenyl)-dimethyl-amine, 9-Isothiocyanato-acridine, 4-Isothiocyanato-quinoline, malachite green isothiocyanate or a derivative thereof.

12. A method according to claim 1 wherein said compound is a ionizable compound or a derivative thereof according to **Figure 4.**

13. A method according to claim 10 wherein said compound is FITC or a derivative thereof.

14. A method according to claim 13 wherein detecting the reacted compound of step d) is performed at a pH above 5, preferably at a pH of about 7.

15. A method according to claim 1 wherein step c) is performed by using size discriminating ultra filtration, preferably followed by an ion exchanging step, or ultracentrifugation, preferably followed by an ion exchanging step.

16. A method according to claim 1 wherein step c) is performed by using LC.

17. A method according to claim 1 wherein step c) is performed by using ion-exchange chromatography.

18. A method according to claim 1 wherein step d) is performed either by using
i) LC or capillary electrophoresis and thereupon illuminating the reacted compound present and measuring the emitted energy or the absorbed energy,
ii) by using MS, preferably preceded by LC, or
iii) a combination of both i) and ii), whereby preferably i) is performed before ii).

19. A method according to claim 18 wherein the illuminating of the reacted compound present and measuring the emitted energy or the absorbed energy is performed using LIF or diode array, preferably LIF.

20. A method according to claim 19 wherein the illumination wavelength is 488 nm (±20 nm) and the measurement of the emitted energy is performed at longer wavelegths.

21. A method according to claim 18 wherein LC or the capillary electrophoresis in i) is followed by transferring the reacted compound present on to a rotary means, preferably a disc, and then illuminating the reacted compound present and measuring the emitted energy or the absorbed energy, whereby the illuminating of the reacted compound present and measuring the emitted energy or the absorbed energy thereof may be performed an unlimited number of times.

22. A method for manufacturing a standard material for use in a method according to any one of claims 1—21 comprising the following steps:
    a) reacting an adduct with a compound comprising a fluorescent or ionizing moiety and
    b) purifying reacted compound by e.g. separating the unreacted compound from reacted compound.

23. A method according to claim 22 wherein said adduct is a globin adduct.

24. A method according to claim 22 wherein said adduct is a hemoglobin adduct.

25. A method according to claim 22 wherein said adduct is a serum albumin adduct.

26. A method according to claim 22 wherein said compound comprises an N=C=S-group (an isothiocyanate-group).

27. A method according to claim 22 wherein said compound is a fluorescein compound or a derivative thereof.

28. A method according to claim 22 wherein said compound is selected from the group FITC, DNITC and DABITC or a derivative thereof.

29. A method according to claim 22 wherein said compound is FITC or a derivative thereof.

30. A standard material obtainable by the method according to any one of claims 23 to 29.

31. Use of a standard material according to claim 30 in a method according to any one of claims 1 – 21.

32. A container for use when analyzing adducts in a fluid or a solid material suspected for containing said adducts, wherein said container comprises means for performing steps a) – c) as set out in claim 1.

33. Use of a method according to any one of claims 1 – 21 for analyzing hazardous substances, such as acryl amide and styrene.

34. Kit comprising standard material according to claim 32.

Abstract

The present invention provides according to a first aspect a method for analyzing adducts in a fluid or a solid material suspected for containing said adducts comprising the following steps:

a) bringing said fluid and/or solid material in direct contact with a isothiocyanate reagent comprising a fluorescent and/or ionizable moiety or a reagent were the isothiocyanate group is directly bounded to an aromatic ring or aromatic ring system, not being unsubstituted phenyl or pentafluorophenyl (i.e., PITC and PFPITC)

b) allowing said compound to react with adducted N-terminal protein/peptide present in said fluid and/or solid material,

c) separating the formed analytes from the reaction mixture, and

d) detecting the analytes.

The present invention also provides according to a second aspect a method for manufacturing a standard material for use in the method according to the first aspect of the present invention comprising the following steps:

a) reacting an N-adducted amino acid or adducted N-terminal terminal peptide/protein with a isothiocyanate compound comprising a fluorescent and/or ionizable moiety and

b) purifying the formed thiohydantoin analyte, e.g., by separating the analyte from the reaction mixture.

The present invention also provides according to a third aspect a standard material obtainable by the method according to the second aspect of the present invention.

The present invention also provides according to a fourth aspect use of a standard material according to the third aspect in a method according to the first aspect of the present invention. The present invention also provides according to a fifth aspect a container for use when analyzing adducts in a fluid or a solid material suspected for containing said adducts, wherein said container comprises means for performing steps a) – c) as set out in the first aspect above. The present invention also provides according to a sixth aspect use of a method according to the first aspect for analyzing hazardous substances, such as acryl amide and styrene. The present invention also

provides according to a seventh aspect a kit comprising standard material according to the third aspect.

Development of a fluorescens-N-*R*-Edman procedure for analysis of protein adducts using LC-MS and/or fluorescens detection.

Hans von Stedingk

Disposition

- Aim
- Background
- Results
  - Synthesis
  - Fluorescence
  - CE
  - HPLC
  - MS
- Discussion

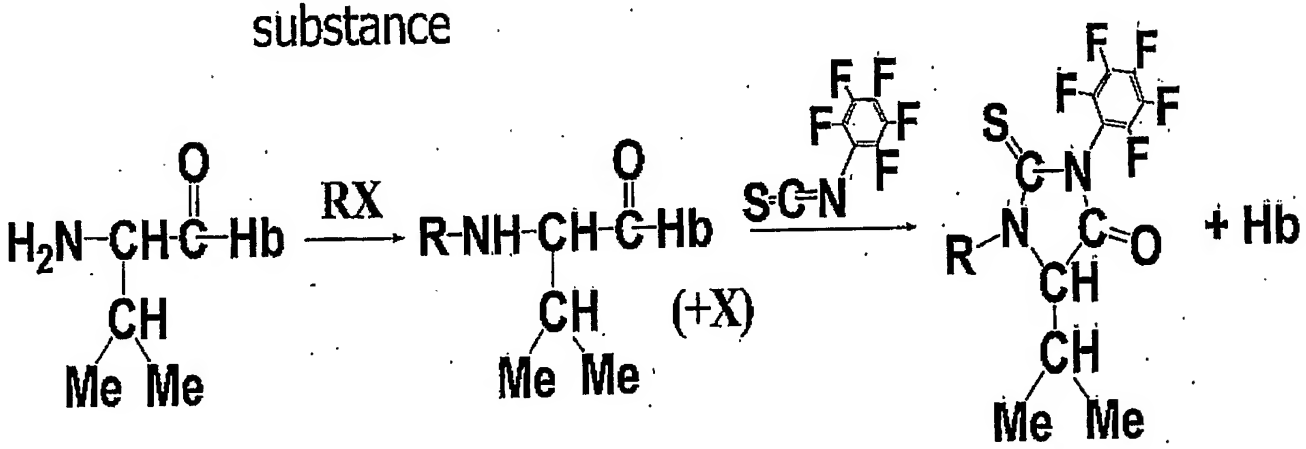
Aim

To develop and evaluate a new method, the so-called FIRE procedure, optimized for e.g., LC-MS/MS analysis and/or analysis with capillary electrophoresis with fluorescence detection.

The N-alkyl Edman method

Reactive electrophilic substance

Derivatisation reagent

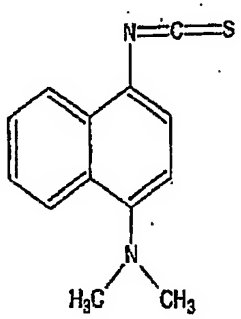


N-terminal valin in heamoglobine

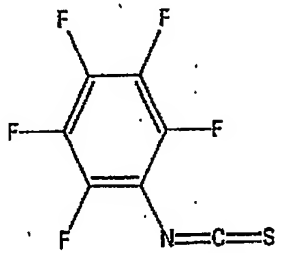
Adduct to valin

The analyte (an isothiohydantoine)

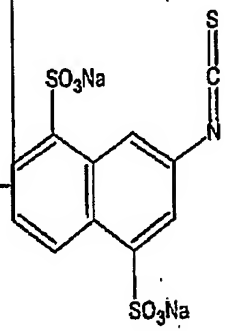
Tested reagents utilized in the FIRE procedure



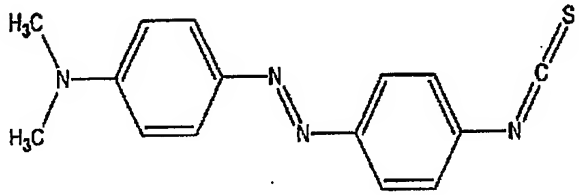
4-N,N-dimethylamino-1-naphthyl isothiocyanate (DNITC)



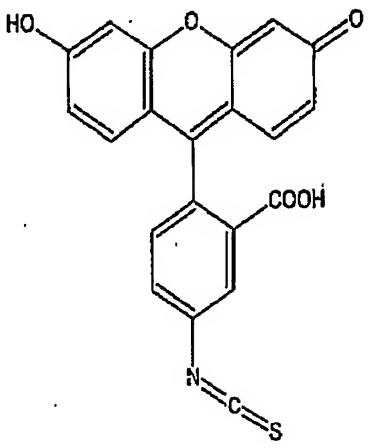
Pentafluorophenyl isothiocyanate (PITC)



Disodium 3-(isothiocyanato)naphthalene -1,5-disulfonate (DSNITC)

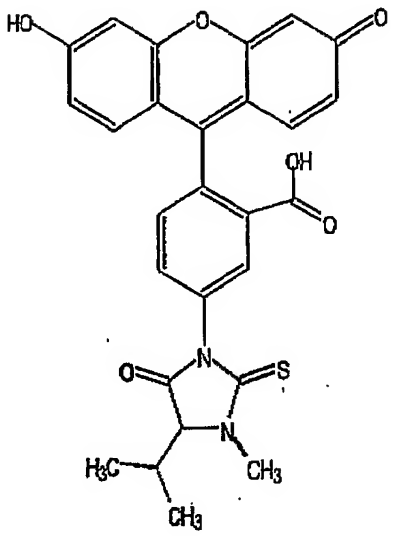


4-N,N-Dimethylaminoazobenzene 4´-isothiocyanate (DABITC)

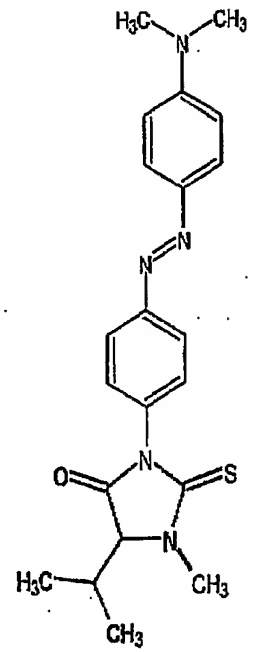


Fluorescein isothiocyanate (FITC)

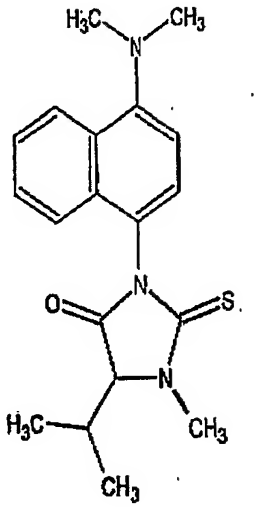
Synthesis



FTH-MeVal



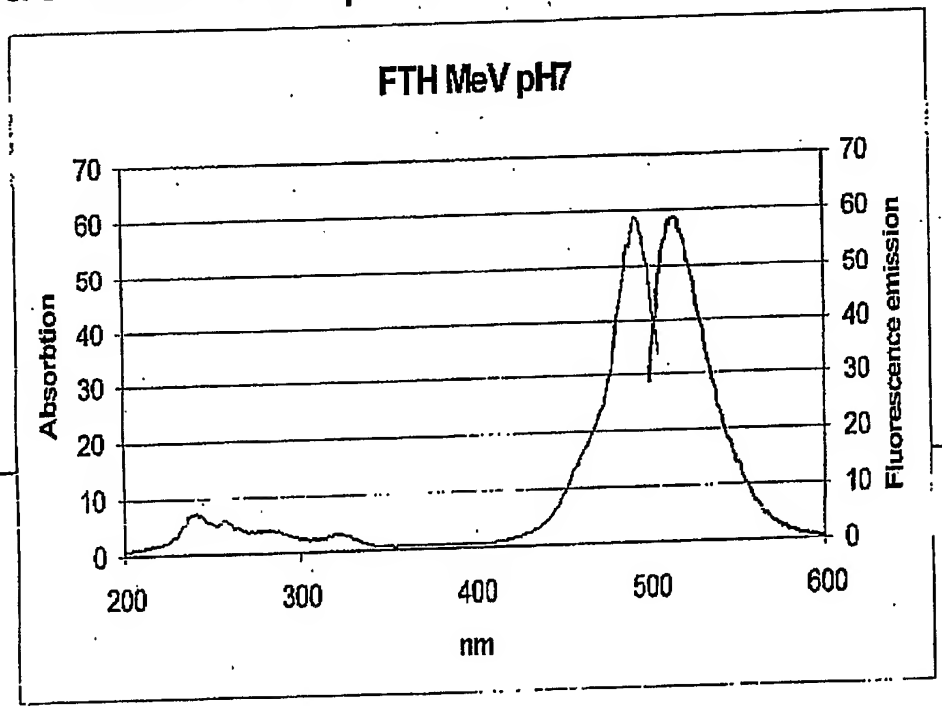
DABTH-MeVal



DNTH-MeVal

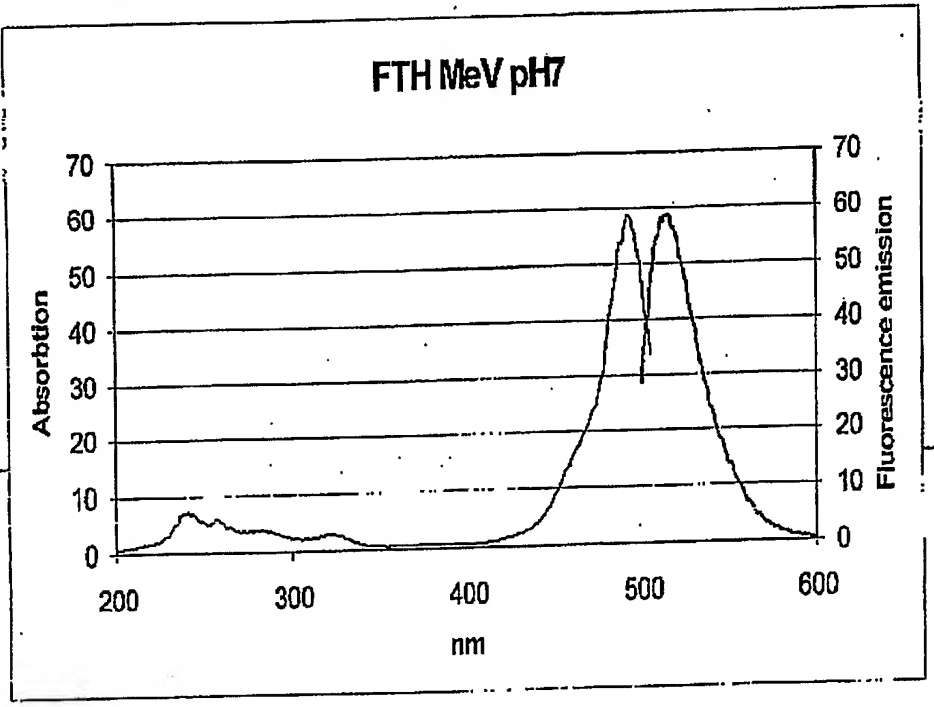
Fluorescens

- Fluorescin had the lowest LOD.
- The adduct doesn't affect the fluorescence spectra.

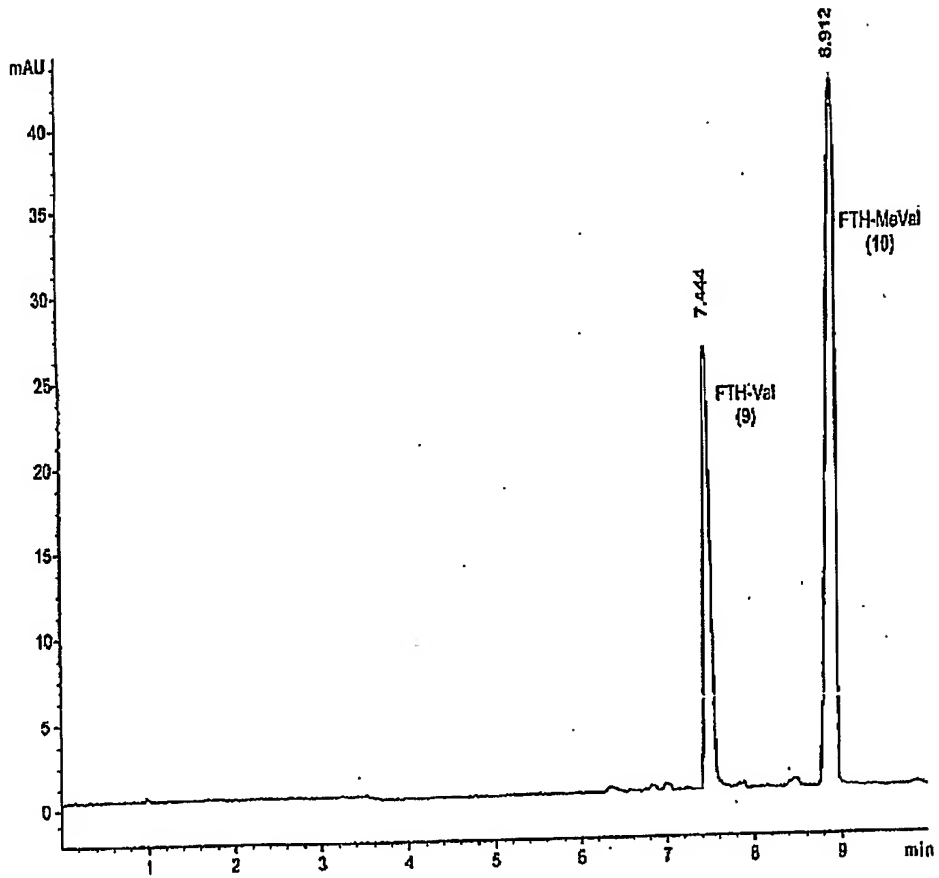


Fluorescens

- Fluorescin had the lowest LOD.
- The adduct doesn't affect the fluorescence spectra.



CE

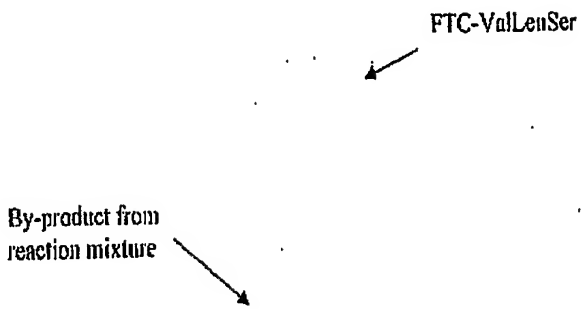


HPLC separation

FTH-MeVal + FTH-Val of pure reference compounds

Formation of FTH-MeVal formed from Fluorescein and MeValLeu-anilide

Reaction between FITC and ValLeuSer with formation of FTC-ValLeuSer

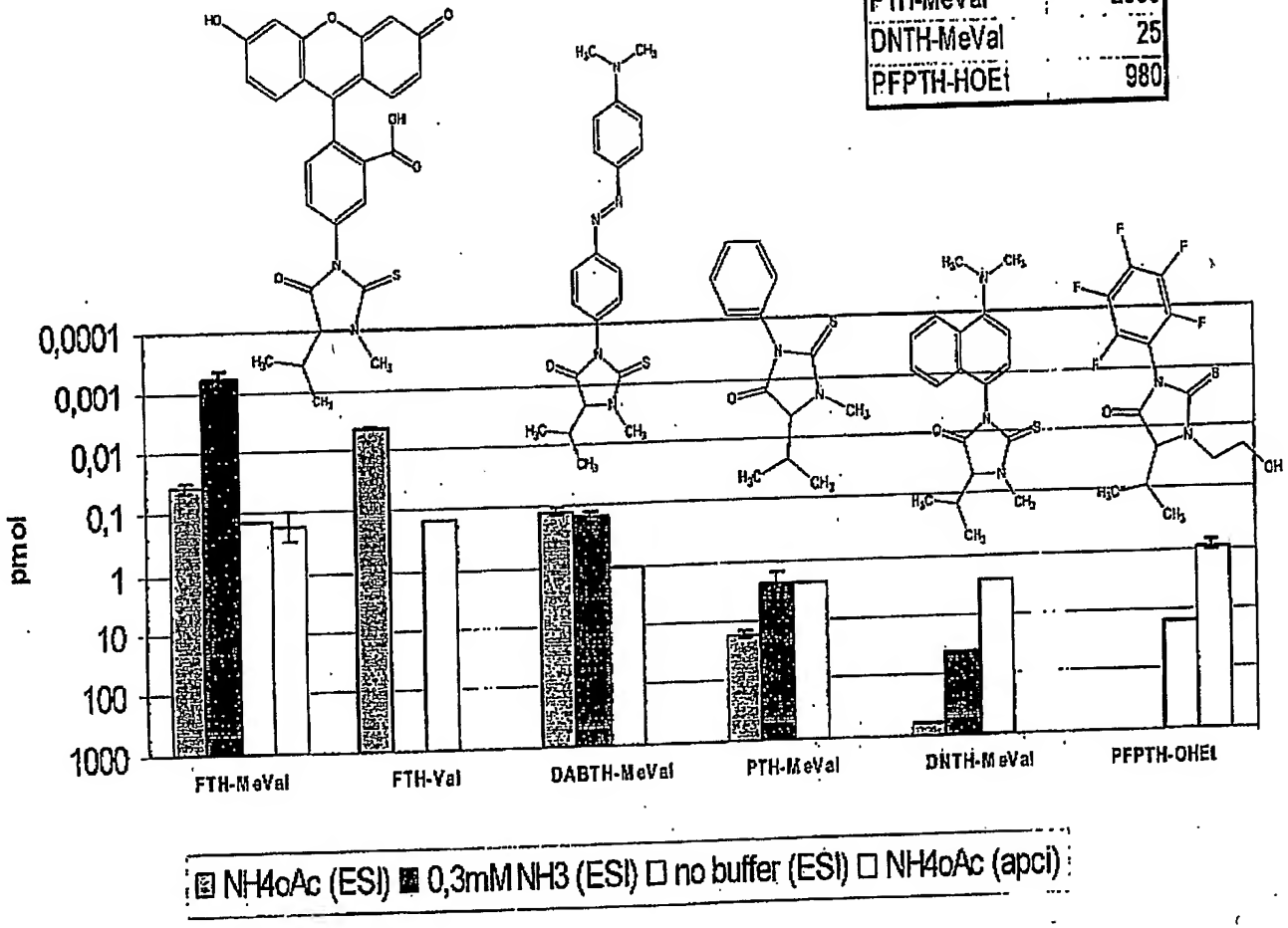


Unsubstituted N-terminals reacts but does not form FTH-Val
In accordance with the N-alkyl Edman procedure.

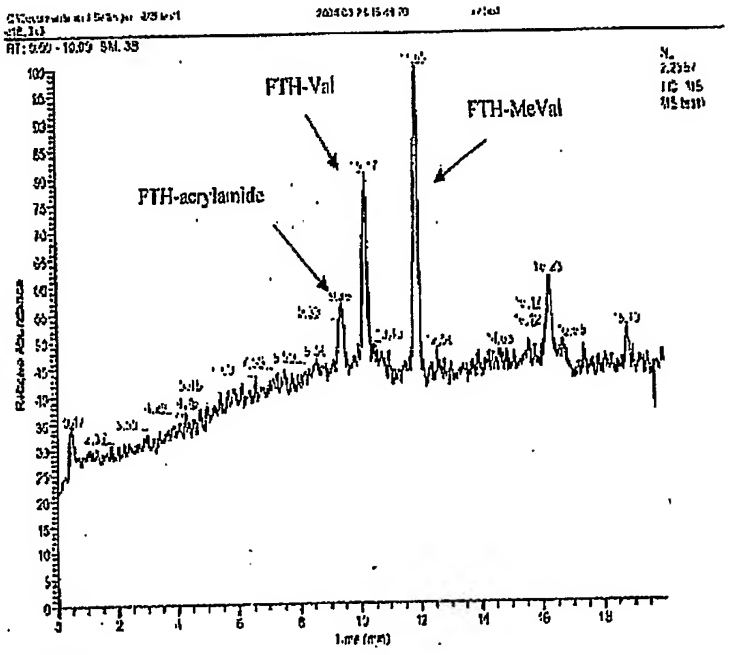
Mass-spectrometry

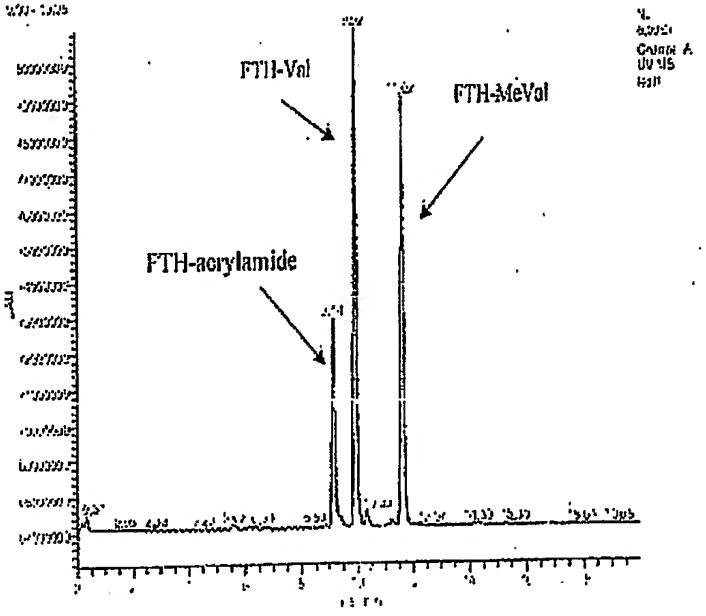
LOD (srm, negative ions)

Relative rating, LOD	
FTH-MeVal	1
FTH-Val	2
DABTH-MeVal	12
PTH-MeVal	2300
DNTH-MeVal	25
PFPTH-HOEt	980



Utilization of the FIRE procedure on alkylated globine





Future perspectives

Opens for:

- Identification of previously unknown adducts.
- Low level of detection.
- Routine analysis, e.g., CE with fluorescence detection

The LC-FI*R*E-procedure

(LC-Fluorescence ionisationable-*R*-Edman procedure.)

The work has been performed with help from:

Per Rydberg (supervisor) and

Jonas Björklund (cosupervisor)

Presentation Tisdag

1. Development of a fluorescence-N-alkyl Edman method for analysis of protein adducts using LC-MS and/or fluorescence detection

2. Disposition

Aim
Background
New reagents
Results
Discussion

Aim

Test and evaluate new reagents for the N-alkyl-Edman method, that make analysis with LC-MS possible and/or analysis on cappilary electrophoresis with fluorescence detection.

The N-alkyl Edman method can be used to predict the dose of an electrofilic compound within the body. With the method it is possible to analyse the adducts that the electrofilic compounds forms to the N-terminal site in haemoglobin.

Bild

The N-alkyl Edman method is used to measure adducts formed to haemoglobin and from that it is possible to predict the dose of an electrofilic compound within the body.

4. The N-alkyl-Edman method

Bild

The N-alkyl-Edman method is specific for adduct affected Hb.

Ej ringslutning normalt...

After the derivated aminoacid has been detached it is possible to analyse the sample with GC-MS. For a successful analysis with GC-MS the substance needs to be quit hydrophobic, relative small and stay intact when it is heated.
If any of these criteria don't match, an other analyse method needs to be used.

6. Nya reagensen...(Varför nya reagens)

7. My work

The first step was to syntesies the expected tiohydantoins from the new reagents. This was made from the aminoacid Valin, both alkylated with methyl and not alkylated. The synthesis of the tiohydantoines worked out find for three of the fuor tested reagents. Rita struktur... The DSNITH was hard to clean up due to its hydrofilicity. We also saw the expected ringclosure for the methylated valin at pH ~8 and we had to use more acid conditions to achive the ringclosure for the not alkylated ones. The formed compounds was caracterised first with TLC and later with nmr and MS. The MS data showed that with had ions with the right molacular mass for each substance.The nmr data indicated that the Fluorescin had an other configuration that was aspected. It wasn't a carboxylic acid.
Rita upp molekylen...I will come back to this later.

The configuration of Fluorescin affects the fluorescent properties and can be regulated with pH. The studies of the flurescent properties for the formed tiohydantoins showed that the Fluorescin tiohydantoins had the lowest level of detection at ph suited for each reagents. The differrens for the methylated and not methylated tiohydantoins didn't had an effect of the fluorescent properties. This is not something that is aspected for other possible adducts either wheil the konjugated system is cut of before the adduct.
Fluorescin showed to be special with a fluorescens spectra with a high excitation wave length of 492 nm. This is something that can be used to detect the compound with an argon laser, with low level of detection.

To achieve a good separation before a fluorescence detection, cappilary electrophoresis can be used.
It was possible to separate the methylated and not methylated FluorescinTH on CE... bild.
(this was detected with UV-detection.)No argon laser were used...DAD.

This means that it is possible to use the reagent on a CE with Argon laser to improve the sensitivity. With this technic: Amino acids have been analysed with this reagent with a LOD of z mol (ten to the power of –21).

For caracterisation of new adducts a good hplc-ms system is needed. The tested reagents showed to be possible to separate with the small difference of a methyl. Ex

Mass-spectrometry
Ms/ms... FTH va bäst... bild The LOD was estimated from the signal to noise data. Only a few attempts were made to optimise the MS system... The table shows the results of LOD relativly to each other with used buffer and measured ion suited for each compound.

The Fluorescin reagent was tested in full scale... Globin that were known to be alkylated with akrylamide were analysed with the new method and analysed with lc-ms. And it worked! bild HPLC-MS/MS test på globin fungerade... this shows that the aa FTH separates from mev+v. Några alkyleringar av större molekyler testades också... men

8. Future perspectivs...

Hopefully the method can be used to identify adducts that hasn't been possible to measure before. Fore this purpuse the hplc-ms properties are crucial. On the other hand the CE-fluorescence detection opens up for analysing adducts at very low levels. And the CE technic is much cheaper which opens up for routine analysis in a bigger scale. What hasn't been taken up jet is that the tested reagents have properties that opens up for a clean up step that isn't available for the traditional reagents. For example has ionexchangers been used in a clean up step for the referenses.

The work will be performed with help from Per Rydberg at the institution of environmental chemestry and Jonas Björklund at the institution of analytic chemistry.

Övrigt... diskussion

Method for analyzing adducts

This invention concerns the technical field of analyzing adducts. In particular the present invention relates to a fluorescent N-*R*-Edman procedure for analysis of N-terminal protein adducts with spectrophotometric and/or mass spectrometric detection methods. Further the invention relates to products necessary in the above mentioned method and uses of said products and said method.

Background

It has been demonstrated earlier that *in vivo* electrophilic compounds can be monitored by measuring the products (adducts) of their reaction with proteins, in particular hemoglobin (Hb) (*1-5*). Important nucleophilic sites in Hb which are reactive under physiological conditions are the imidazole nitrogen atoms in histidine residues, sulfur atoms in cysteine and methionine residues, oxygen atoms in carboxyl groups and in hydroxyl groups in tyrosine and serine residues, and the α-nitrogen atoms in the N-terminal valine residue of all four chains of human Hb (*6*).

The so called N-alkyl Edman procedure was developed for measurements of adducts (mainly low molecular weight adducts) to N-terminal valine residues in Hb (*7*). This method was based on the original Edman degradation procedure (*8,9*) used for protein sequencing . It was observed that N-terminal valine N-alkylated with a radioactively labelled 2-hydroxyethyl moiety from ethylene oxide was released spontaneously as a phenylthiohydantoin (PTH) under the conditions (pH >7) employed for the coupling reaction between phenyl isothiocyanate (PITC) and protein. The released PTH could be separated from unmodified N-terminal valine residues, as well as from the rest of the protein by extraction.

This observation led to the development of the N-alkyl Edman procedure for mass spectrometric (MS) quantification of Hb adducts (*10*). Because of its usefulness, the N-alkyl Edman method has been applied in a number of laboratories for research purposes, dose monitoring and hygienic surveillance (*11-16*).

A brief description of the N-alkyl Edman procedure is presented in Figure 1. A sample of the globin (isolated from red blood cells by acid precipitation) is dissolved in formamide and pentafluorophenyl isothiocyanate (PFPITC) is then added, together with a small amount of aqueous 1 M NaOH in order to obtain a near neutral solution. The mixture is maintained at room temperature overnight, after which the temperature is raised to 45 °C for a couple of hours (*17*). The pentafluorophenylthiohydantoin (PFPTH) derivative of the terminal N-alkylvaline residues are released in high yield by this procedure and subsequently isolated by extraction.

Although the N-alkyl.Edman procedure has become an established method for analysis of N-substituted haemoglobin adducts, the method is connected by limitations, e.g., the range of adducts that can be analyzed. Small adducts, e.g., ethylene oxide and propylene oxide can be quantified at nmol/g globin level, which is sensitive enough for measurement of so called background adduct levels (levels without known exposure). However, adducts with a few polar groups are more difficult to measure, mainly because of elution problems in the gas chromatographic (GC) system prior to the MS detection. Some of these limitations can be solved, e.g., by further derivatisation (Paulsson et al), this approach is both time consuming and demands introduction of new steps to be developed for each specific adduct. Adducts with; high molecular weigh (>700 mass units), many polar groups and/or are thermo labile will be extremely difficult to analyze with the GC-MS based N-alkyl Edman procedure.

In order to overcome one or more of these problems and to optimize the sensitivity and increase the applicability range the "Fluorescent N-*R*-Edman procedure", i.e. the present method, is provided. This invention is based on the principles of the original N-alkyl Edman procedure, alkylated N-terminals can be detached and measured as their corresponding thiohydantoin derivatives after coupling with isothiocyanate Edman reagents.

Summary of the Invention

The present invention solves one or more of the above problems by providing according to a first aspect a method for analyzing adducts in a fluid or a solid material suspected for containing said adducts comprising the following steps:

a) bringing said fluid and/or solid material in direct contact with a compound comprising a fluorescent and an ionizing moiety,

b) allowing said compound to react with adducts present in said fluid and/or solid material,

c) separating the un-reacted compound from the reacted compound; and

d) detecting the reacted compound.

The present invention also provides according to a second aspect a method for manufacturing a standard material for use in the method according to the first aspect of the present invention comprising the following steps:

a) reacting an adduct with a compound comprising a fluorescent or ionizing moiety and

b) purifying reacted compound by e.g. separating the unreacted compound from reacted compound.

The present invention also provides according to a third aspect a standard material obtainable by the method according to the second aspect of the present invention.

The present invention also provides according to a fourth aspect use of a standard material according to the third aspect in a method according to the first aspect of the present invention. The present invention also provides according to a fifth aspect a container for use when analyzing adducts in a fluid or a solid material suspected for containing said adducts, wherein said container comprises means for performing steps a) – c) as set out in the first aspect above. The present invention also provides according to a sixth aspect use of a method according to the first aspect for analyzing hazardous substances, such as acryl amide and styrene. The present invention also provides according to a seventh aspect a kit comprising standard material according to the third aspect.

Accordingly hereby a novel method is introduced for analysis of electrophilic compounds measured *in vivo* as their corresponding adducts by means of the so-called "Fluorescent N-*R*-Edman procedure". This invention is based on the original observation that N-alkylated N-terminal protein adducts are detached with high selectivity from adducted proteins as their corresponding N-alkyl-valine phenylthiohydantoines after derivatisation with phenyl isothiocyanate (PITC) or pentafluorophenyl isothiocyanate (PFPITC) in the so called N-alkyl Edman procedure. In the Fluorescent N-*R*-Edman procedure fluorescent Edman reagents are preferably used (i.e. the isothiocyanates; FITC , DNITC and DABITC), detached as their corresponding thiohydantoines which has been isolated from tested N-substituted amino acids model peptides and adducted protein. The analytes may be separated on both liquid chromatography (LC) and capillary electrophoresis (CE) and detected by e.g. mass spectrometry (MS) and fluorescence spectroscopic techniques. Low limit of detection (LOD) was obtained using LC-MS/MS techniques (low fmol level on a standard instrument) and apotency for measurements down to low zmol (10^{-21}) levels utilizing CE-laser induced fluorescence (LIF) analysis.

Due to the mild and non-discriminating conditions utilized for the Fluorescens N-*R*-Edman procedure its applicability range is wider than for all earlier existing methods for measurements of N-terminal protein adducts. This new method have excellent sensitivity and specificity, the range for measurement spans from adducts with low molecular masses to adducts that are thermo labile, have high molecular weight and/or have high polarity.

This new method should have the potency for routine analysis for hygienic surveillance, medical purpose and for analysis of forensic substances as their *in vivo* formed electrophillically reactive metabolites.

In contrast to the N-alkyl Edman procedure, this method is based on liquid chromatographic systems, e.g. LC and capillary electrophoresis (CE) techniques with MS and/or fluorescence spectroscopic detection methods. Due to the mild and non-discriminating conditions utilized for the Fluorescens N-*R*-Edman procedure its applicability range is much wider compared to the N-alkyl Edman method. The introduction of fluorescent

isothiocyanate reagents provides new possibilities for adduct measurements e.g. capillary electrophoresis with laser induced fluorescence detection (CE-LIF, ref) and HPLC with fluorescence detection for N-terminal protein adducts. The use of fluorescent isothiocyanates has been used for peptide sequencing (*18,19*) at nmolar levels by use of e.g., fluorescein isothiocyanate in combination by CE-LIF (*21*), in the study performed by Ireland *et al* the LOD were established to be on the low zmol level.

The Fluorescent N-*R*-Edman procedure is further improved by the utilization of ionisable groups in the applied reagents, e.g., tertiary amines and carboxyl groups, which provides possibilities to enrich the analytes on ion-exchangers, facilitates CE separation and also delivers higher affinity for ionisation in the MS, providing higher sensitivity. The principles of the Fluorescent N-*R*-Edman procedure is presented in Figure 2.

Detailed description of the invention

It is intended throughout the present description that the expression "LC" embraces any kind of liquid chromatography, but preferably it is HPLC i.e. High Pressure Liquid Chromatography.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein said adduct has been formed involving a secondary N-terminal valine.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein said adduct is a globin adduct.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein said adduct is a hemoglobin adduct.

According to a preferred embodiment of the first aspect of the invention there is provided a method, wherein said compound comprises an N=C=S-group (an isothiocyanate-group).

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein said compound is a fluorescein compound or a derivative thereof.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein said compound is selected from the group FITC, DNITC and DABITC or a derivative thereof.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein said compound is FITC or a derivative thereof. The FITC may be 4' or 5'.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein detecting the reacted compound of step d) is performed at a pH above 5, preferably at a pH of about 7.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein step c) is performed by using size discriminating ultra filtration, preferably followed by an ion exchanging step, or ultracentrifugation, preferably followed by an ion exchanging step. Instead of ultracentrifugation or size discriminating ultra filtration it would be plausible to use osmotic principles to separate the un-reacted from the reacted compounds. When FITC or a derivative thereof is used in said method, an anion exchanger is preferably used in the ion exchanging step and when DNITC or DABITC or a derivative thereof is used an anion exchanger is preferably used in the ion exchanging step.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein step c) is performed by using LC.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein step c) is performed by using ion-exchange chromatography.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein step d) is performed either by using

i) LC or capillary electrophoresis and thereupon illuminating the reacted compound present and measuring the emitted energy or the absorbed energy,

ii) by using MS, preferably preceded by LC, or

iii) a combination of both i) and ii), whereby preferably i) is performed before ii).

Preferably a standard material according to the second aspect of the invention is run first in the above method whereby i), ii) or a combination of both is used, which enables that a first scan can show where the searched adduct would appear if present in the analyte. By using this standard material first it possible to get a hint of where the adduct should appear during the detection step.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein the illuminating of the reacted compound present and measuring the emitted energy or the absorbed energy is performed using LIF or a diode array, preferably LIF.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein the illumination wavelength is 288 nm and the measurement of the emitted energy is performed at 520 nm.

According to a preferred embodiment of the first aspect of the invention there is provided a method wherein LC or the capillary electrophoresis in i) is followed by transferring the reacted compound present on to a rotary means, preferably a disc, and then illuminating the reacted compound present and measuring the emitted energy or the

absorbed energy, whereby the illuminating of the reacted compound present and measuring the emitted energy or the absorbed energy thereof may be performed an unlimited number of times.

According to a preferred embodiment of the second aspect of the invention there is provided a method wherein said adduct is a globin adduct.

According to a preferred embodiment of the second aspect of the invention there is provided a method wherein said adduct is a hemoglobin adduct.

According to a preferred embodiment of the second aspect of the invention there is provided a method wherein said compound comprises an N=C=S-group (an isothiocyanate-group).

According to a preferred embodiment of the second aspect of the invention there is provided a method wherein said compound is a fluorescein compound or a derivative thereof.

According to a preferred embodiment of the second aspect of the invention there is provided a method wherein said compound is selected from the group FITC, DNITC and DABITC or a derivative thereof.

According to a preferred embodiment of the second aspect of the invention there is provided a method wherein said compound is FITC or a derivative thereof.

Preferred features of each aspect of the invention are as for each of the other aspects mutatis mutandis. The prior art documents mentioned herein are incorporated to the fullest extent permitted by law. The invention is further described in the following examples in conjunction with the appended figures, which do not limit the scope of the invention in any way. Embodiments of the present invention are described in more detail with the aid of examples of embodiments and figures, the only purpose of which is to illustrate the invention and are in no way intended to limit its extent.

Short description of the figures

Fig. 1 shows a brief description of the N-alkyl Edman procedure.

Fig. 2 shows the principles of the Fluorescent N-*R*-Edman procedure, i.e the present method.

Fig. 3 shows the reagents for the N-alkyl Edman procedure, phenyl isothiocyanate (PITC) and pentafluorophenyl isothiocyanate (PFPITC) which were evaluated towards three fluorescent isothiocyanate reagents; fluorescein isothiocyanate (FITC), 4-N,N-dimethylaminoazobenzene 4'-isothiocyanate (DABITC) and 4-dimethylamino-1-naphthyl isothiocyanate (DNITC).

Fig. 4 shows structures of compounds 1-14 as set out in the example part of the present description.

Fig. 5 shows an NMR obtained (see example part).

Fig. 6 shows ^{13}C NMR spectra of FTH-MeVal,

Fig. 7 shows ^{1}H NMR spectra of FTH-MeVal

Fig. 8 shows ^{13}C NMR spectra of FTH-MeVal.

Fig. 9 - 12 show results from the study in the exemplary part.

Fig. 13 shows results form fluorescence measurements.

Fig. 14 - 20 show results from another study in the exemplary part.

Fig. 21 shows a complete baseline separation that was obtained.

Fig. 22 shows studied thiohydantoin analytes.

Fig. 23 shows limit of Detection (LOD), measured with LC-MS/MS, positive ions, different pH, data presented on a log scale.

Fig. 24 shows limit of Detection (LOD), measured with LC-MS/MS, negative ions, different pH, data presented in a log scale.

Fig. 25 shows a comparison of fluorescence properties for the analysed compounds. FTHMeVal gives lowest LOD. Fluoranten is used as a reference. The excitation and emission spectra are measured with wavelength and pH suited for each compound.

Fig. 26 shows excitation and emission spectra of FTH Val (9) and FTH-MeVal (10). The emission spectra was recorded at ex: 492 nm and the emission at em: 515 nm. The FTH Val (9) and FTH-MeVal (10) where measured at the same conditions and concentration (pH~7; 0.1μM; ACN:H_2O (1:4)).

Fig. 27 shows LC-MS/MS analysis of acrylamide adducted human globin by use of the Fluorescent N-*R*-Edman procedure and comparison with reference compounds.

Examples

In order to compare the Fluorescent N-*R*-Edman procedure with the reagents that are used in the original N-alkyl Edman method and also recently has been applied for LC-MS/MS analysis (Ph.D., Hubert Vesber, using PFPITC, precented in Anaheim, CA May 2004, at the ACS meeting, acrylamide section), a comparative study was performed. The reagents for the N-alkyl Edman procedure, phenyl isothiocyanate (PITC) and pentafluorophenyl isothiocyanate (PFPITC) were evaluated towards three fluorescent isothiocyanate reagents; fluorescein isothiocyanate (FITC), 4-N,N-dimethylaminoazobenzene 4'-isothiocyanate (DABITC) and 4-dimethylamino-1-naphthyl isothiocyanate (DNITC) (see Figure 3). The tested reagents were reacted with valine and N-methyl valine, models for non-substituted N-terminal globin and N-substituted N-terminal valine in globine, *N*-(2-carbamoylethyl)-valine were reacted with PFPITC. The formed analytes; phenyl-(valin and N-methylvaline)thiohydantoin (PTH-Val and PTH-MeVal), pentafluorophenyl-*N*-(2-carbamoylethyl)-valine thiohydantoin (PFPTH-AAVal), fluorescein-(valin and N-methylvaline)thiohydantoin (FTH-Val and FTH-MeVal), 4'-N,N-dimethylaminoazobenzene-4-

(valin and N-methylvaline)thiohydantoin (DABTH-Val and DABTH-MeVal) and 4-dimethylamino-1-(valin and N-methylvaline)thiohydantoin (DANTH-Val and DANTH-MeVal) were then compared by measurements at various pH on UV, HPLC and LC-MS/MS. The fluorescent analytes were also measured on fluorescence spectroscopy (excitation and emission spectra) and on CE with diode array detection. As it was observed that one of the selected fluorescent reagent were superior in comparison with the rest of the reagents, this reagent were also evaluated on alkylated model peptides and globine adducted with acrylamide (AA), glycidamide (GA), propylene oxid (PE), cholesterol-5α,6α-epoxide (Chol-EO) and 2-octadecyl-oxirane.

Experimental

Material and Methods

Chemicals. Structures of compounds 1-14 are given in Figure 4.Fluoresceine isothiocyanate (isomer I, <90%), pentafluorophenyl isothiocyanate (PFPITC) and phenyl isothiocyanate (PITC, purum) were obtained from Fluka. 4-N,N-Dimethylaminoazobenzene 4'-isothiocyanate (DABITC) and 4-dimethylamino-1-naphthyl isothiocyanate (DNITC) were obtained from Acros. Cholesterole-5α,6α-epoxide, octadecyl-epoxide, L-Valine (Val), L-valinamide ($ValNH_2$), *N*-methyl-*D,L*-valine (MeVal) and 5-isopropyl-3-phenyl-2-thiohydantoin (Val-PTH, 1) were obtained from Sigma. *N*-Methylvalylleucylanilide (MeValLeu-NHφ > 99 %) and valylleucylserine [ValLeuSer (H-Val-Leu-Ser-OH) 95%) were obtained from Bachem (Bubendorf, Switzerland). ($^2H_3$)Acetonitrile (99.8 % ^2H), (^2H)chloroform (99.8 % ^2H), deuterium oxide (99.9 % ^2H, ^2H_2O), and ($^2H_4$)methanol (99.8 % ^2H) were obtained from CIL (Andover, MA). 5-Isopropyl-1-methyl-3-phenyl-2-thiohydantoin (MeVal-PTH, 6), 5-isopropyl-3-pentafluorophenyl-2-thiohydantoin (Val-PFPTH, 12) and 5-isopropyl-1-methyl-3-pentafluorophenyl-2-thiohydantoin (MeVal-PFPTH, 13) were synthesised as described earlier (*20*). Glycidamide (GA) were synthesised. All other chemicals and solvents were of analytical grade.

Instrumentation, methods for analysis and characterization. ^1H and ^{13}C NMR spectra were recorded on a JEOL GSX 270 instrument at 270 MHz. All solvents used were fully deuterated, TMS was added as internal standard in chloroform, acetonitrile and methanol.

Methods and instrumentation for LC-MS/MS analysisis: The LC-MS system comprized a Rheos 4000LC pump (Flux Instruments, Basel, Switzerland) interfaced with a LCQ (ThermoQuest, CA, USA). The MS was operated in electrospray ionisation (ESI) mode. The mobile phase consisted of 1:1, H_2O:acetonitrile at an isocratic flow at 200 µL/min. The ion-source temperature was 120 °C, capillary voltage 3.5 kV and the cone voltage varied between 25 and 140 V. Nitrogen was used as drying gas at a flowrate of 250 L/h. Both the

positive- and the negative-ion mode were used. MS-MS was performed by utilizing collision-induced dissociation (CID) of the [M+1] ion.

Studies of retention times of compounds 4-10 were carried out on HPLC, on a Shimadzu LC-4A connected with a Kromasil LC-18 column (250 x 10 mm) and a Shimadzu SPD-2AS detector (λ = 268 nm, D_2 lamp). Flow rate = 2.5 mL/min, loop 0.7 ml eluted with 2 % aqueous acetonitrile buffered with 0.02 % TFA.

TLC was performed using silica gel 60 f-254 plates (SiO_2, Merck), spots were developed with UV and at long wave (378 nm) Melting points were determined on a Büchi 535 instrument. Mikrokemi AB, Uppsala, Sweden, performed elementary analyses. Measurements of pH were carried out on an Orion EA 920 pH-meter equipped with a Ross 8130 glass electrode.

The CE separation was performed on an HP 3D CE with a five channel diode array UV detector (Agilent, CA, USA). A fused silica capillary (i.d. 50 µm, o.d. 375 µm) with a total length of 64 cm and an effective length of 56 cm was used. The separation voltage was +30 kV, resulting in an separation current of 32 µA. The buffer system consisted of a 17 mM phosphate buffer (adjusted to pH 7) containing 20 mM SDS.

Synthesis of N-Me(4-Me_2N-Naftyl)valin-tiohydantoin (5, DMAP-MeVal). D,L-MeVal (prepared according to Rydberg *et al* 93, 93.0 mg, 0,708 mmol) was alkalized with KOH (0,4 mmol) and dissolved in 0,5 M $KHCO_3$ (3 ml) and dioxane (2 ml). The solution was heated to 45 °C with mágnetic stirring and p-dimethylnafthylisothiocyanate (DNITC, 91.3 mg, 0.40 mmol) dissolved in dioxane (2 ml) was added and the solution. The reaction was followed on TLC (SiO_2, toluene and 2:1 toluene / ethyl acetate) with a blank reference (DNITC dissolved in the solvent mixture). After 60 min the reaction was completed, no DMNaf-ITC could be detected on TLC and the formed product gave fluorescence at long wave under the UV-lamp. The reaction was extracted with toluene and purified by column chromatography ((SiO_2, toluene and 2:1 toluene / ethyl acetate), to yield 130 mg, 96 % yield. The product was re-crystallized from ethanol/water (1:1), to yield white crystals (100 mg, 73 % yield), for NMR, see Figure 5.

Synthesis of 4-Me_2N-Naftyl)valin-tiohydantoin (4, DMAP-Val). L-Valine (Sigma, 804 mg, 6.86 mmol) was alkalized with KOH (3.43 mmol) and dissolved in 0,5 M $KHCO_3$ (3 ml) and dioxan (3 ml). The solution was heated to 45 °C with magnetic stirring and DMNaf-ITC (88 mg, 0.385 mmol) dissolved in dioxane (2 ml) was added to the suspended solution. The reaction was followed on TLC (SiO_2, Ethyl acetate / MeOH 4:1, toluene and 2:1 toluene / ethyl acetate) with a reference whithout addition of Valine. After 60 min the reaction was in principal completed, no DMNaf-ITC could be detected on TLC and the formed product, the 4-Me_2N-Naftyl)-tiocarbamoyl-valinate gave fluorecense at long wave under the UV-lamp (tailing spot on TLC eluted with Ethyl acetate / MeOH 4:1). The reaction was acidified with

hydrochloric acid (3 ml conc.) and the cyclisation was interrupted after 2 h, at 45 ° by neutralization with solid $KHCO_3$ until no more carbodioxide was formed. The reaction mixture was diluted with water (100 ml) and the product was purified by extraction with $CHCl_3$ (125 ml). The organic phase was dried with Na_2SO_4, filtered and evaporated to yield 126 mg, yield 0.96 % as a white solid.

The synthesis of compound 6-14 were performed as described above, the products were confirmed by ^1H and ^{13}C NMR spectra and by LC-MS/MS (ESI) positive and negative ionization, eg., Figure 6, ^{13}C NMR spectra of FTH-MeVal, Figure 7, ^1H NMR spectra of FTH-MeVal and Figure 8, ^{13}C NMR spectra of FTH –MeVal.

Results

In order to evaluate the potency of the tested isothiocyanates the relative response of compound 1-14 (see Figure 3) were measured and compared using fluorescence spectroscopy (compound 4-10), HPLC, CE and UV spectroscopy. For the main part of these studies pH were alternated below and above the pKa for the respective analyte (for pKa values see Figure 4). The studies on relative fluorescence were performed on the fluorescent analytes (compound 4-10 in Figure 4) by measurements of excitation and emission spectra. The results from this study is presented in figure 9-12, FTH-Val and FTH-MeVal gave both in principle identically and high response at pH above 5, showing that the adduct, e.g., the methyl in FPT-MeVal (9) does not effect the spectroscopic properties in comparison with FTH-Val (8). In these studies FTH-Val and FTH-Me Val gave superior response compared to the other used reagents.

In order to evaluate the potency of the tested isothiocyanates the relative response of compound 1-14 (see Figure 3) were measured and compared using UV spectroscopy, HPLC, CE and fluorescence spectroscopy (compound 4-10). For the main part of these studies pH were alternated below and above the pKa for the respective analyte (for pKa values see Figure 4). The studies on relative fluorescence were performed on the fluorescent analytes by measurements of excitation and emission spectra. The results from this study is presented in figure 9-12, FTH-Val and FTH-MeVal gave both in principle identically and high response at pH above 5, showing that the adduct, e.g., the methyl in FPT-MeVal (9) does not effect the spectroscopic properties in comparison with FTH-Val (8). In these studies FTH-Val and FTH-Me Val gave superior response compared to the other used reagents.

In order to compare the relative sensitivity on LC-MS/MS compound 1-9 were compared by injecting 5 µl directly in the electrospray interface of the TSQ (n=3 for each compound), the cone voltage were adjusted in order to obtain maximum sensitivity for the each specific analyte. The LC-MS/MS(ESI) measurements were performed using well

adopted buffer systems, e.g., 0.1 %TFA, 0.1 % ammonium acetate and 0.3 mM aqueous ammonia. For LOD measurements the flow rate were 200 µl/min of the aqueous buffer/acetonitrile [1:1 (v/v)] the analytes were dissolved in the same buffers and solvent mixtures at concentrations ranging from 10 µg/ml down to 1 ng/ml depending on their response for each used condition. The results from this study are summarized in Figures 14-20 and in Table 1.

Table 1. Comparison of relative sensitivities obtained by measurements of limit of detection detection LOD on LC-MS/MS(ESI), using un-buffered or pH modified solvents. LOD were meaured on positive and negative ions with direct injections (5 ul loop). The highest sensitivity (lowest LOD) was obtained with FTH-MeVal, negative ion mode, 0.3 mM NH_3 (marked yellow/dashed) and was set to one.

Measured ions Used buffer/pH Modifier	Positive ion 0,1% NH_4OAc	Positive 0,1% TFA	Positive 0,3 mM NH_3	Positive No pH modifier	Negative 0,1% NH_4OAc	Negative 0,3mM NH_3	Negative No pH modifier
(measured pH)	(pH~7)	(pH~1)	(pH~9-10)		(pH~7)	(pH~9-10)	
FTH-MeVal	15,8	3,9	4,6	447,6	13.8	1.0[a]	50,1
FTH-Val[b]	3,6	1,1	No data	393,1	1,6	No data	57,3
DABTH-MeVal	60,4	26.9	9,8	377.7	45.1	55.9	386.7
DNTH-MeVal	940.8	90,9	460,7	932,1	216593,1	15752,2	984,8
PTH-MeVal[c]	14439,5	7821,4	594.1	36098,9	6618,1	902,5	895.3

Footnotes: [a] LOD (defined as three times signal to noise) calculated to be 2.6 fmol - (multiplication of given values with 2.6 gives LODs in fmol). [b]FTH-Val used as a control for non methylated valines. [c]Corresponding pentafluorinated compound to PTH-MeVal, gave higher LODs than PTH-MeVal, data not presented).

As compound 8 and 9, FTH-Val and FTH-MeVal, gave superior response under the tested conditions for the LC-MS analysis in combination with excellent column separation on HPLC (C_{18}-colums, especially when eluted at acidic conditions), they were selected for further studies. In order to investigate the applicability range for this reagent, valine and globine were adducted with glycidamide, propylene oxide and the high molecular weight adducts cholesterole-5α,6α-epoxide and octadecyl-epoxide. The formed thiohydantoines (compounds 10-14) were characterized on LC-MS/MS and the adducted globins could be measured after derivatisation (90 min in 0.5 M aqueous $KHCO_3$/ 2-propanol [2/1 (v/v)], followed by size discriminating ultra filtration (MWCO 5000), evaporation filtrate or concentration of the analytes from the filtrate by use of anion exchangers. This approach is not only time saving but is also non-discriminating especially for analysis of adducts with high

polarity as they often are lost or gives poor yields on liquid/liquid or solid phase extraction clean up steps.

In order to evaluate the possibility to measure N-terminal adducts with such sensitive techniques as CE-LIF, the separation between F-MeValTH and F-ValTH on CE were evaluated. A complete baseline separation was obtained using the stated conditions e.g., in 17 mM phosphate buffer (adjusted to pH 7) containing 20 mM SDS (see Figure 21). Separation between FTH-Val (9) and FTH-MeVal (10) on Capillary electrophoresis was measured with diode array detection and FTH-Val elutes at 7.44 min and FTH MeVal elutes at 8.91 min (phosphate buffer 17 mM, pH 7, 20 mM SDS). 30 kV, 52 cm capillary, 1 nl injected. Accordingly, in order to evaluate the possibility to measure N-terminal adducts with such sensitive techniques as CE-LIF, the fluorescent analytes, 5-10, were also measured with fluorescence spectroscopy (excitation and emission spectra) and analyzed on CE with diode array detector (UV). The separation between FTH-Val (9) and FTH-MeVal (10) on CE was evaluated as set out eearlier. A complete baseline separation was obtained as set out above (Figure 21). The intensity of the fluorescence reaches an optimum around this pH for both FTH-Val and FTH-MeVal, which opens up the possibility to utilize CE-LIF for adducts measurements down to the low zmol (10^{-21}) levels, which has been shown by Ireland *et.al.* (*21*). In their study 18 of 20 coded FTH-amino acids were separated and analysed with LOD of around 10 zmol..

To summarize, selected methylated and non-methylated analytes, (1, 2; 5, 6; 7, 8 and 9, 10 see Figure 22; N.B. other numbering than in Figure 4) were synthesized and compared using different analytical methods, e.g., by measurements with fluorescence spectroscopy, LC-UV, CE-DAD and LC-MS/MS under different conditions and pH ranges. It was observed that fluorescein isothiocyanate (FITC) was superior, regarding separation, solubility, sensitivity on MS and spectroscopic properties in comparison to the other reagent candidates. Thus, FITC was selected for further studies using alkylated model peptides and globine adducted with acrylamide (11), glycidamide (12), 2-octadecyl-oxirane (13), propylene oxide (14), cholesterol-5α,6α-epoxide, (15). These analytes (11-15, Figure 22; N.B. other numbering than in Figure 4) were analysed on LC-MS/MS(ESI) and were shown to give good response in the applied system. Further on, it was possible to isolate these adducts from alkylated globin derivatized with FITC, by size discriminating ultra filtration (MWCO 5000) followed by anion exchanger. This approach is not only time saving but is also non-discriminating especially for analysis of adducts with high polarity as they often are lost or gives poor yields on liquid/liquid or solid phase extraction clean up steps. The isolated extracts were then directly analyzed on LC-MS/MS successfully with a high sensitivity. Data that show the sensitivity for the tested reagents and the corresponding methylated and not methylated tiohydantoins are also presented in Table 1 (above) and in Figure 23-24.

Studies on relative fluorescence were performed on the fluorescent analytes (compound 5-10) by measurements of excitation and emission spectra. The result from this study is presented in Figure 25. The results show that FTH-MeVal (10) gave detectable response at the lowest concentration. FTH-MeVal (10) was detected at a concentration 360 times lower compared to Fluoranten, which was used as a reference. In comparison to the other analytes FTH-MeVal (10) was measured at concentrations 45 times lower than DABTH-MeVal (6) and 530 times lower than DNTH-MeVal.(8) The excitation and emission spectra were measured at wavelengths and pH suited for each compound.

In these studies FTH-Val (9) and FTH-Me Val (10) gave superior response compared to the other used reagents. These compounds also have specific excitation and emission at wavelengths around 492 and 515 nm respectively (figure 26). That make them suitable for excitation using an Argon-ion laser (488 nm). Both FTH-Val (9) and FTH-MeVal (10) gave similar high response (at pH above 5), showing that the adduct, e.g., the methyl in FPT-MeVal (10) does not effect the spectroscopic properties in comparison with FTH-Val (9). This opens up the possibility to utilize CE-LIF (Lacer Induced Fluorecence) for adducts measurements down to the low zmol (10^{-21}) levels (FTH-amino acids measured), which has been shown by Ireland *et. al. (21)*. In their study 18 of 20 coded FTH-amino acids, formed according to the original Edman method (22), were separated and analyzed with LOD of around 10 zmol. These results are truly encouraging for miniaturizing the fluorescent-N-*R* Edman procedure, aiming to measure adduct spectra from a few µl blood, easily available by a prick in the fingertip

It was further possible to separate the corresponding tiohydantoins, methylated and non-methylated, for all four tested reagents on LC. Different gradients were used for the different reagents and, in spite of the small structural differences between the methylated and the non-methylated molecules, baseline separations were achieved. A chromatogram of the LC-MS separation is presented in Figure 27, were the FTH-acrylamide adduct (11), FTH-MeVal (10) and FTH-MeVal (9) are separated on LC and identified with mass-spectrometry. The experiment was performed on acrylamide alkylated human globin and corresponding FTH-adducts (9-11; see figure 22) as references.

References

1. Ehrenberg, L., Hiesche, K. D., Osterman-Golkar, S. and Wennberg, I. (1974) Evaluation of genetic risks of alkylating agents: Tissue doses in the mouse from air contaminated with ethylene oxide. *Mutat. Res.* 24, 83-103.

2. OstermanGolkar, S., Ehrenberg, L., Segerbäck, D. and Hällström, I. (1976) Evaluation of genetic risks of alkylating agents. II. Haemoglobin as a dose monitor. *Mutat. Res.* 34, 1-10.

3. Neumann, H. G., Baur.H and Wirsing.R. (1980) Dose-response relationship in the primary lesion of strong electrophilic carcinogens. *Arch. Toxicol. Suppl.* 3, 69-77.

4. Skipper, P. L., and Tannenbaum, S. R. (1990) Protein adducts in the molecular dosimetry of chemical carcinogens. *Carcinogenesis* 11, 507-518.

5. Törnqvist, M., Fred, C., Haglund, J., Helleberg, H., Paulsson, B. and Rydberg, P. (2002) Protein adducts: quantitative and qualitative aspects of their formation, analysis and applications. *J. Chromatogr. B* 778, 279-308.

6. Osterman-Golkar S, and Ehrenberg, L. (1980) Alkylation of macromolecules for detecting mutagenic agents.*Teratog.Carcinog.Mutag.* 1, 105-127.

7. Jensen, S., Törnqvist, M., and.Ehrenberg, L. (1984) Hemoglobin as a dose monitor of alkylating agents: Determination of alkylation products of N-terminal valine. (F.J.de Serres and R.W.Pero, Ed.) pp 315-320, Plenum Press, New York

8. Edman, P. (1950) Methods for determination of the amino acid sequence in peptides. *Acta Chem. Scand.* 4, 283-298.

9. Edman, P., and Henschen, A. (1975) Sequence determination. In *Protein Sequence Determination* (Needleman S. B., Ed.) pp 232-279, Springer-Verlag, Berlin

10. Törnqvist, M., Mowrer, J., Jensen, S. and Ehrenberg, L. (1986) Monitoring of environmental cancer initiators through hemoglobin adducts by a modified Edman method. *Anal. Biochem.* 154, 255-266.

11. Tates, A. D., Grummt, T., Törnqvist, M., Farmer, P. B., van Dam, F. J., van Mossel, H., Schoemaker, H. M., Osterman-Golkar, S., Uebel, C., Tang, Y. S., Zwinderman, A. H., Natarajan, A. T. and Ehrenberg, L. (1991) Biological and chemical monitoring of occupational exposure to ethylene oxide. *Mutat. Res.* 250, 483-497.

12. Törnqvist, M., Magnusson, A. L., Farmer, P. B., Tang, Y. S., Jeffery, A. M., Wazneh, L., Beulink, G. D. T., van der Waal, H. and van Sittert, N. J. (1992) Ring test for low-levels of *N*-(2-hydroxyethyl)valine in human hemoglobin. *Anal. Biochem.* 203, 357-360.

13. Bergmark, E., CALLEMAN, C. J., He, F., and Costa, L. G. (1993) Hemoglobin adducts in humans occupationally exposed to acrylamide.*Toxicol.Appl.Pharmacol.* 120, 45-54.

14. Fidder, A., Noort, D., de Jong, A. L., Trap, H. C., de Jong, L. P., and Benschop, H. P. (1996) Monitoring of in vitro and in vivo exposure to sulfur mustard by GC/MS determination of the N-terminal valine adduct in Hb after a modified Edman degradation.*Chem.Res.Toxicol.* 9, 788-792.

15. Thier, R., Lewalter, J., Kempkes, M., Selinski, S., Bruning, T., and Bolt, H. M. (1999) Haemoglobin adducts of acrylonitrile and ethylene oxide in acrylonitrile workers,

dependent of polymorphisms of the glutathione transferases GSTT1 and GSTM1. *Occup.Environ.Med.* 56, 197-202.

16. Begemann, P., Sram, R. J. and Neumann, H. G. (2001) Hemoglobin adducts of epoxybutene in workers occupationally exposed to 1,3-butadiene. *Arch. Toxicol.* 74, 680-687.
17. Törnqvist, M. (1994) Epoxide adducts to N-terminal valine of hemoglobin. *Methods Enzymol.* (Everse J., Vandergriff K. D., and Winslow R. W., Eds.) pp 650-657, Academic Press, New York
18. Edman, P. (1950) Methods for determination of the amino acid sequence in peptides. *Acta Chem. Scand.* 4, 283-298.
19. Edman, P., and Henschen, A. (1975) Chapter Title (Needleman, S. B.) pp Chap 8, Springer-Verlag, Berlin and New York
20. Rydberg, P., Lüning, B., Wachtmeister, C. A. and Törnqvist, M. (1993) Synthesis and characterization of *N*-substituted valines and their phenyl-thiohydantoins and pentafluorophenyl- thiohydantoins. *Acta Chem. Scand.* 47, 813-817.
21. Ireland, I. D., Lewis, D. F., Li, X., Renborg, A., Kwong, S., Chen, M., and Dovichi, N. J. (1997) Duoble Coupling Edman Chemistry for High-sensitivity Automated Protein Sequencing. *J. Protein Chem.* 16, 491-493.
22. Edman, P. (1950) Methods for determination of the amino acid sequence in peptides. *Acta Chem. Scand.* 4, 283-298.

Various embodiments of the present invention have been described above but a person skilled in the art realizes further minor alterations, which would fall into the scope of the present invention. The breadth and scope of the present invention should not be limited by any of the above-described exemplary embodiments, but should be defined only in accordance with the following claims and their equivalents. For example, any of the above-noted methods can be combined with other known methods. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

Claims

1. Method for analyzing adducts in a fluid or a solid material suspected for containing said adducts comprising the following steps:
   a) bringing said fluid and/or solid material in direct contact with a compound comprising a fluorescent and an ionizing moiety,
   b) allowing said compound to react with adducts present in said fluid and/or solid material,
   c) separating the un-reacted compound from the reacted compound, and
   d) detecting the reacted compound.

2. A method according to claim 1 wherein said adduct has been formed involving a secondary N-terminal valine.

3. A method according to claim 1 wherein said adduct is a globin adduct.

4. A method according to claim 1 wherein said adduct is a hemoglobin adduct.

5. A method according to claim 1 wherein said compound comprises an N=C=S-group (an isothiocyanate-group).

6. A method according to claim 1 wherein said compound is a fluorescein compound or a derivative thereof.

7. A method according to claim 1 wherein said compound is selected from the group FITC, DNITC and DABITC or a derivative thereof.

8. A method according to claim 7 wherein said compound is FITC or a derivative thereof.

9. A method according to claim 8 wherein detecting the reacted compound of step d) is performed at a pH above 5, preferably at a pH of about 7.

10. A method according to claim 1 wherein step c) is performed by using size discriminating ultra filtration, preferably followed by an ion exchanging step, or ultracentrifugation, preferably followed by an ion exchanging step.

11. A method according to claim 1 wherein step c) is performed by using LC.

12. A method according to claim 1 wherein step c) is performed by using ion-exchange chromatography.

13. A method according to claim 1 wherein step d) is performed either by using
i) LC or capillary electrophoresis and thereupon illuminating the reacted compound present and measuring the emitted energy or the absorbed energy,
ii) by using MS, preferably preceded by LC, or
iii) a combination of both i) and ii), whereby preferably i) is performed before ii).

14. A method according to claim 13 wherein the illuminating of the reacted compound present and measuring the emitted energy or the absorbed energy is performed using LIF or diode array, preferably LIF.

15. A method according to claim 14 wherein the illumination wavelength is 288 nm and the measurement of the emitted energy is performed at 520 nm.

16. A method according to claim 13 wherein LC or the capillary electrophoresis in i) is followed by transferring the reacted compound present on to a rotary means, preferably a disc, and then illuminating the reacted compound present and measuring the emitted energy or the absorbed energy, whereby the illuminating of the reacted compound present and measuring the emitted energy or the absorbed energy thereof may be performed an unlimited number of times.

17. A method for manufacturing a standard material for use in a method according to any one of claims 1 – 16 comprising the following steps:
a) reacting an adduct with a compound comprising a fluorescent or ionizing moiety and
b) purifying reacted compound by e.g. separating the unreacted compound from reacted compound.

18. A method according to claim 18 wherein said adduct is a globin adduct.

19. A method according to claim 18 wherein said adduct is a hemoglobin adduct.

20. A method according to claim 18 wherein said compound comprises an N=C=S-group (an isothiocyanate-group).

21. A method according to claim 18 wherein said compound is a fluorescein compound or a derivative thereof.

22. A method according to claim 18 wherein said compound is selected from the group FITC, DNITC and DABITC or a derivative thereof.

23. A method according to claim 23 wherein said compound is FITC or a derivative thereof.

24. A standard material obtainable by the method according to any one of claims 18 to 24.

25. Use of a standard material according to claim 25 in a method according to any one of claims 1 – 17.

26. A container for use when analyzing adducts in a fluid or a solid material suspected for containing said adducts, wherein said container comprises means for performing steps a) – c) as set out in claim 1.

27. Use of a method according to any one of claims 1 – 17 for analyzing hazardous substances, such as acryl amide and styrene.

28. Kit comprising standard material according to claim 26.

Abstract

The present invention provides according to a first aspect a method for analyzing adducts in a fluid or a solid material suspected for containing said adducts comprising the following steps:

a) bringing said fluid and/or solid material in direct contact with a compound comprising a fluorescent and an ionizing moiety,
b) allowing said compound to react with adducts present in said fluid and/or solid material,
c) separating the un-reacted compound from the reacted compound, and
d) detecting the reacted compound.

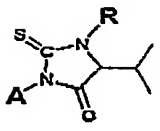
The present invention also provides according to a second aspect a method for manufacturing a standard material for use in the method according to the first aspect of the present invention comprising the following steps:

a) reacting an adduct with a compound comprising a fluorescent or ionizing moiety and
b) purifying reacted compound by e.g. separating the un-reacted compound from reacted compound.

The present invention also provides according to a third aspect a standard material obtainable by the method according to the second aspect of the present invention.

The present invention also provides according to a fourth aspect use of a standard material according to the third aspect in a method according to the first aspect of the present invention. The present invention also provides according to a fifth aspect a container for use when analyzing adducts in a fluid or a solid material suspected for containing said adducts, wherein said container comprises means for performing steps a) – c) as set out in the first aspect above. The present invention also provides according to a sixth aspect use of a method according to the first aspect for analyzing hazardous substances, such as acryl amide and styrene. The present invention also provides according to a seventh aspect a kit comprising standard material according to the third aspect.

Fig. 22/27.



Analyte	(nr)	Adduct	A (used reagent)
PTH-Val	(1)	R=H	PITC
PTH-MeVal	(2)	R=Me	PITC
PFPTH-MeVal	(3)	R=Me	PFPITC
PFPTH-HOEtVal	(4)	R=CH_2CH_2OH	PFPITC
DABTH-Val	(5)	R=H	DABITC
DABTH-MeVal	(6)	R=Me	DABITC
DNTH-Val	(7)	R=H	DNITC
DNTH-MeVal	(8)	R=Me	DNITC

Analyte	(nr)	Adduct	A = FITC
FTH-Val	(9)	R=H	(used in all reactions)
FTH-MeVal	(10)	R=Me	
FTH-AAVal	(11)	R=$CH_2CH_2CONH_2$	
FTH-GAVal	(12)	R=$CH_2CH(OH)CONH_2$	
FTH-HOC$_8$Val	(13)	R=$CH_2CH(OH)[(CH2)_5CH_3$	
FTH-HOPrVal	(14)	R=$CH_2CH(OH)CH_3$	
FTH-CholEO-Val	(15)	R=$C_{27}H_{45}O_3$	

(adduct from cholesterol-5α,6α-epoxide)

Figure 22. Studied thiohydantoin analytes.

Fig. 23/27.

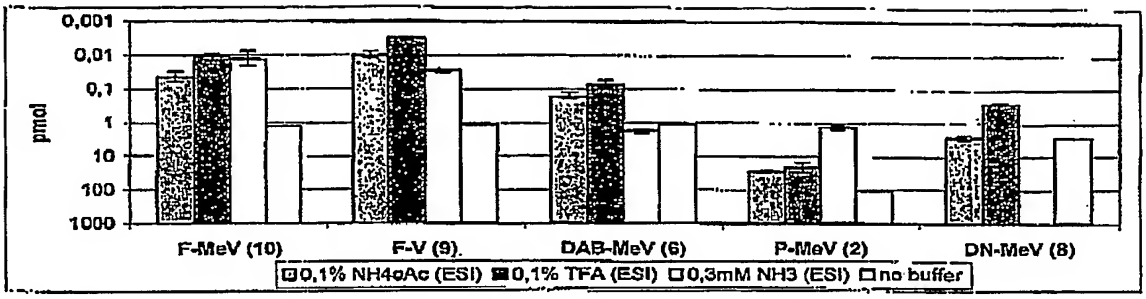


Figure 23. Limit of Detection (LOD), measured with LC-MS/MS, positive ions, different pH, data presented on a log scale.

Fig.24/27

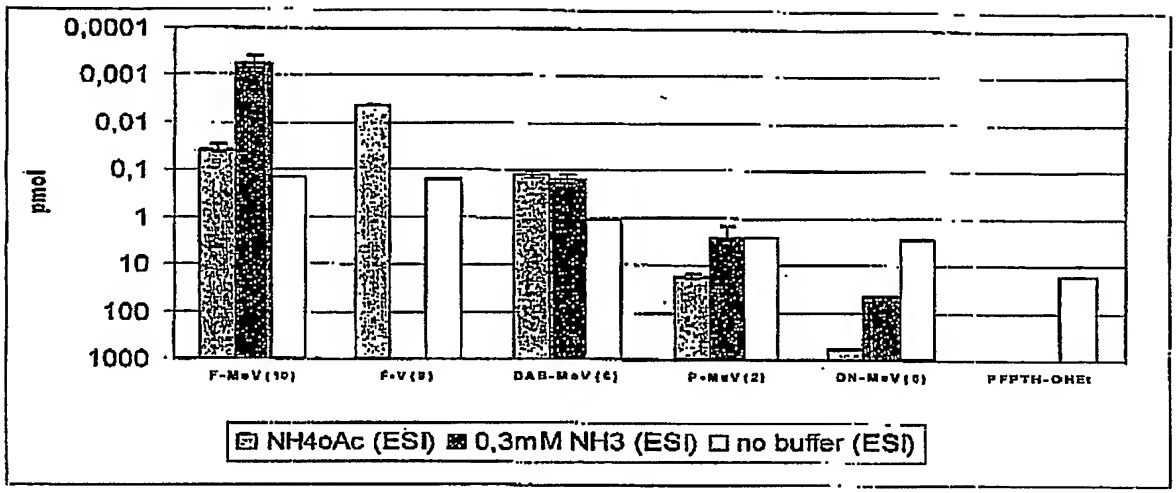


Figure 24. Limit of Detection (LOD), measured with LC-MS/MS, negative ions, different pH, data presented in a log scale.

Fig. 25/27.

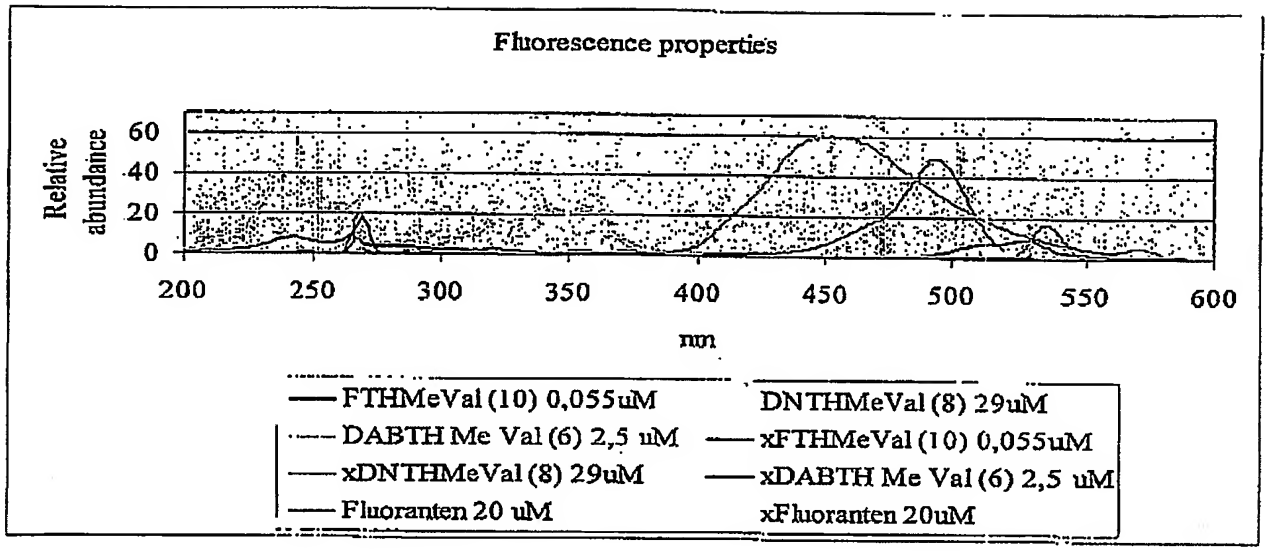


Figure 25: Comparison of fluorescence properties for the analysed compounds. FTHMeVal gives lowest LOD. Fluoranten is used as a reference. The excitation and emission spectra are measured with wavelength and pH suited for each compound.

Fig. 26/27

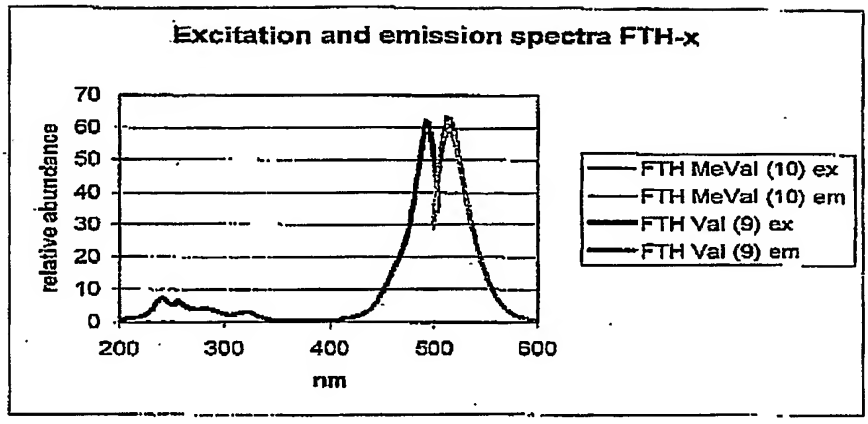


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Figure 26: Excitation and emission spectra of FTH Val (9) and FTH-MeVal (10). The emission spectra was recorded at ex: 492 nm and the emission at em: 515 nm. The FTH Val (9) and FTH-MeVal (10) where measured at same conditions and concentration (pH~7; 0,1μM; ACN:H_2O (1:4)).

Fig. 27/27.

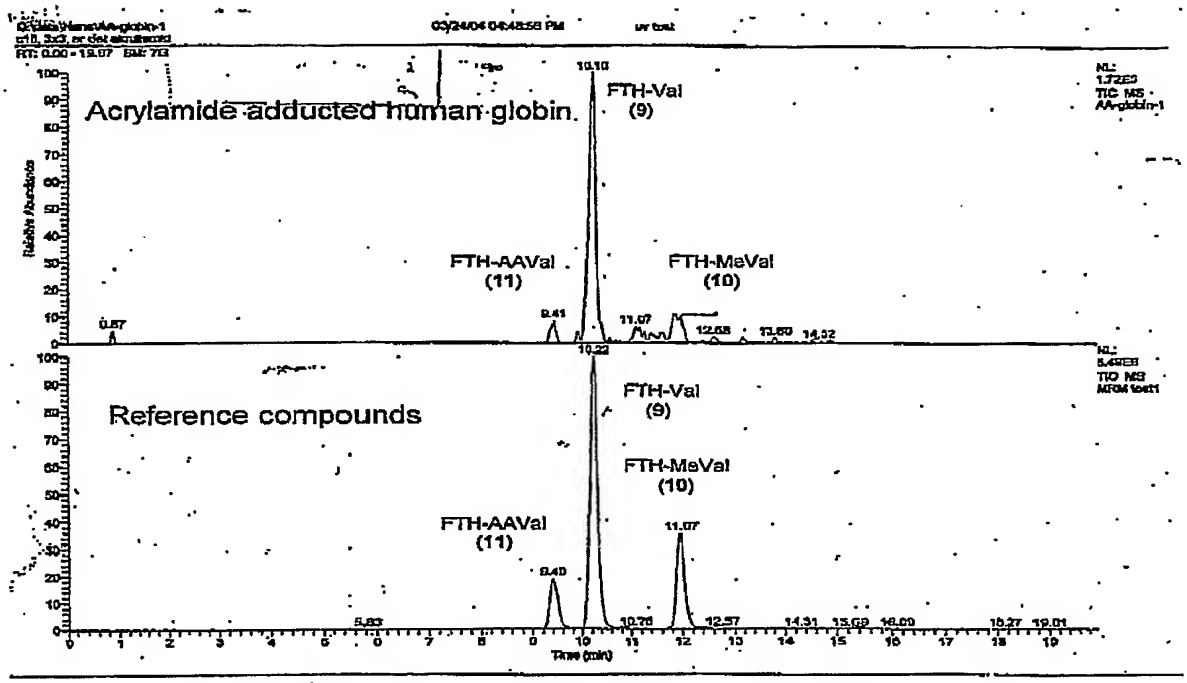
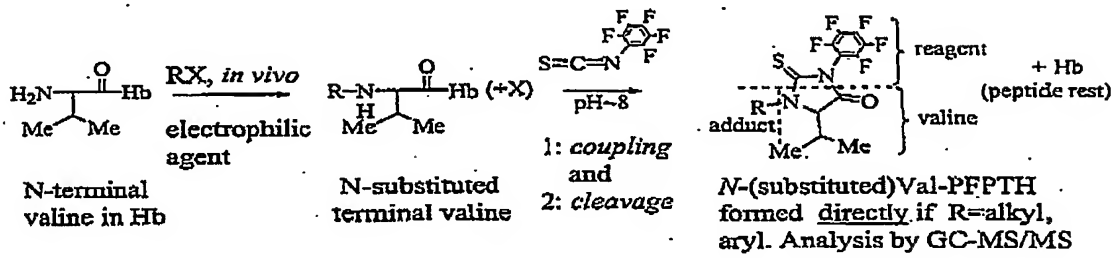


Figure 27. LC-MS/MS analysis of acrylamide adducted human globin by use of the Fluorescent N-*R*-Edman procedure and comparison with reference compounds.

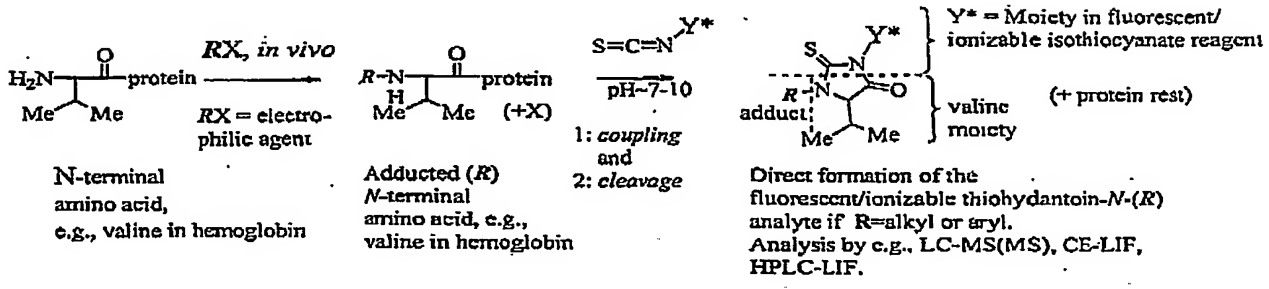
Figures:

Figure 1. Principles of the N-alkyl Edman procedure[a].



Footnote: [a]Phenyl isothiocyanate and pentafluorophenyl isothiocyanate have been utilized in this procedure.

Figure 2. Principles of the fluorescent/ionizable N-*R*-Edman (FIRE) procedure



N-terminal amino acid, e.g., valine in hemoglobin

Adducted (*R*) *N*-terminal amino acid, e.g., valine in hemoglobin

Direct formation of the fluorescent/ionizable thiohydantoin-*N*-(*R*) analyte if R=alkyl or aryl. Analysis by e.g., LC-MS(MS), CE-LIF, HPLC-LIF.

Figure 3. Studied reactions, coupling between selected isothiocyanate reagents (Y-N=C=S) and valine/adducted valine (*R*=H or alkyls) on amino acid (X=O⁻), peptide (X=LeuSer) and protein level (X=rest of human globin).

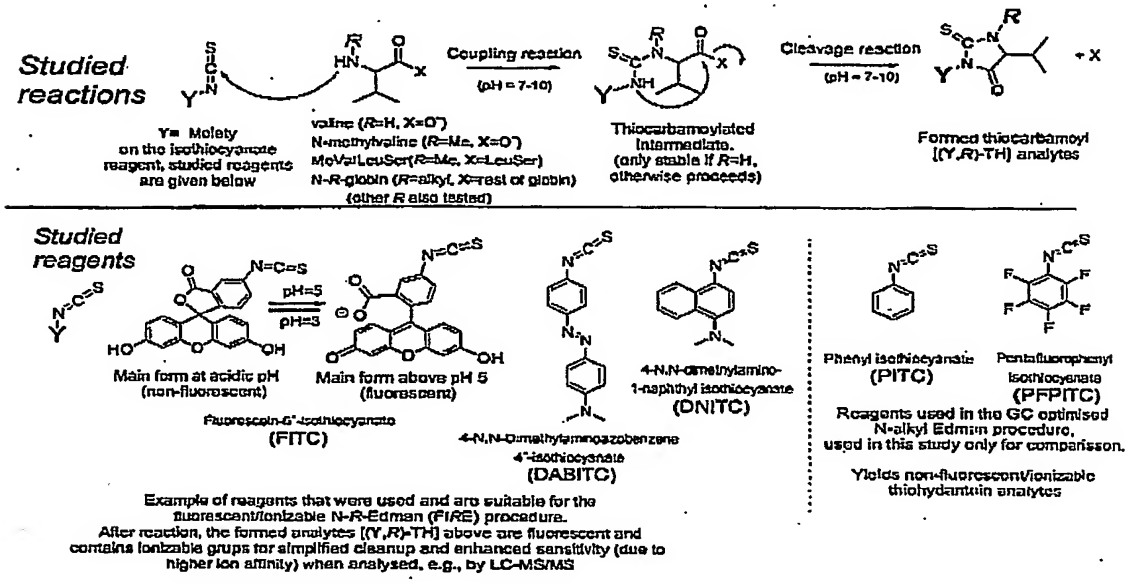
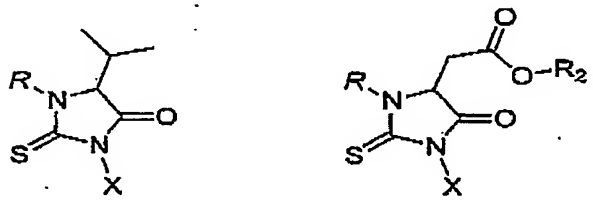


Figure 4. General structure of adducted analytes and proposed adducted analytes formed in the FIRE procedure from N-terminal valine e.g. in proteins such as globine (XTH-*R*-Val)[a] and analytes formed from N-terminal asparagines, e.g., in proteins such as Bovine serum albumin (XTH-R-Asp-R_2)[b].

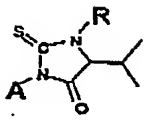


XTH-*R*-Val XTH-*R*-Asp-R_2

Footnotes: [a]The R substituent to the valine thiohydantoin represents any adduct (e.g., alkyl and aryl or substituted homologues of alkyl and aryl, but not hydrogen) covalently bounded to the valine nitrogen. The X substituent to the valine thiohydantoin represents the moiety of any utilized isothiocyanate reagent in which the isothiocyanate group is directly bounded to an aromatic ring or an aromatic ring system providing fluorescent and/or ionisable and/or other valuable properties to the analyte, however, X is not phenyl and pentafluorophenyl, e.g., PITC and PFPITC.

[b]The R substituent to the asparagine thiohydantoin represents any adduct (e.g., alkyl and aryl or substituted homologues of alkyl and aryl, but not hydrogen) covalently bounded to the valine nitrogen. The X substituent to the asparagine thiohydantoin represents the moiety of any utilized isothiocyanate reagent in which the isothiocyanate group is directly bounded to an aromatic ring or an aromatic ring system providing fluorescent and/or ionisable and/or other valuable properties to the analyte, however, X is not phenyl and pentafluorophenyl, e.g., PITC and PFPITC. The R_2 substituent to the carboxyl group of asparagine represents; hydrogen, alkyl, aryl, carboxyl, bensyl or substituted analogues to these, this carboxyl group can also be an anion.

Figure 5. Studied thiohydantoin analytes.



Analyte	(nr)	Adduct	A (used reagent)
PTH-Val	(1)	R=H	PITC
PTH-MeVal	(2)	R=Me	PITC
PFPTH-MeVal	(3)	R=Me	PFPITC
PFPTH-HOEtVal	(4)	$R=CH_2CH_2OH$	PFPITC
DABTH-Val	(5)	R=H	DABITC
DABTH-MeVal	(6)	R=Me	DABITC
DNTH-Val	(7)	R=H	DNITC
DNTH-MeVal	(8)	R=Me	DNITC

Analyte	(nr)	Adduct	A = FITC
FTH-Val	(9)	R=H	(used in all reactions below)
FTH-MeVal	(10)	R=Me	
FTH-AAVal	(11)	$R=CH_2CH_2CONH_2$	
FTH-GAVal	(12)	$R=CH_2CH(OH)CONH_2$	
FTH-HOC$_{11}$Val	(13)	$R=CH_2CH(OH)(CH_2)_nCH_3$	
FTH-HOPrVal	(14)	$R=CH_2CH(OH)CH_3$	
FTH-CholEO-Val	(15)	$R=C_{27}H_{45}O_2$ (adduct from cholesterol-5α,6α-epoxide)	

Figure 6. Comparison of relative sensitivities, presented in log scale, obtained by measurements of limit of detection detection LOD on LC-MS/MS in ESI and in APCI mode.

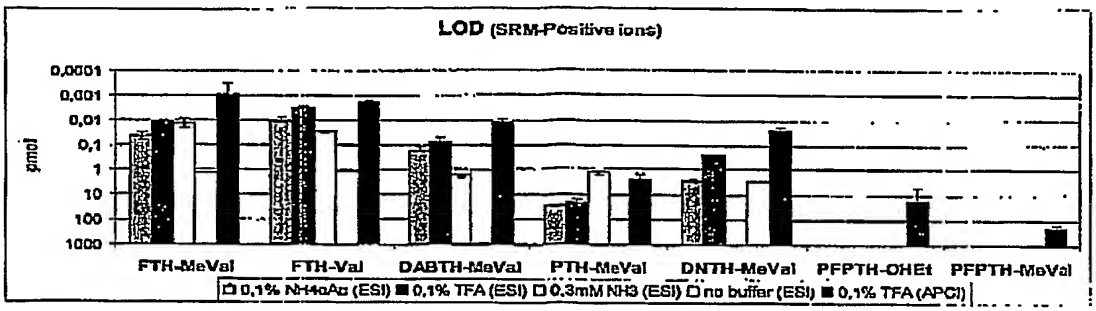


Figure 7. Comparison of relative sensitivities, presented in log scale, obtained by measurements of limit of detection detection LOD on LC-MS/MS in ESI and in APCI mode

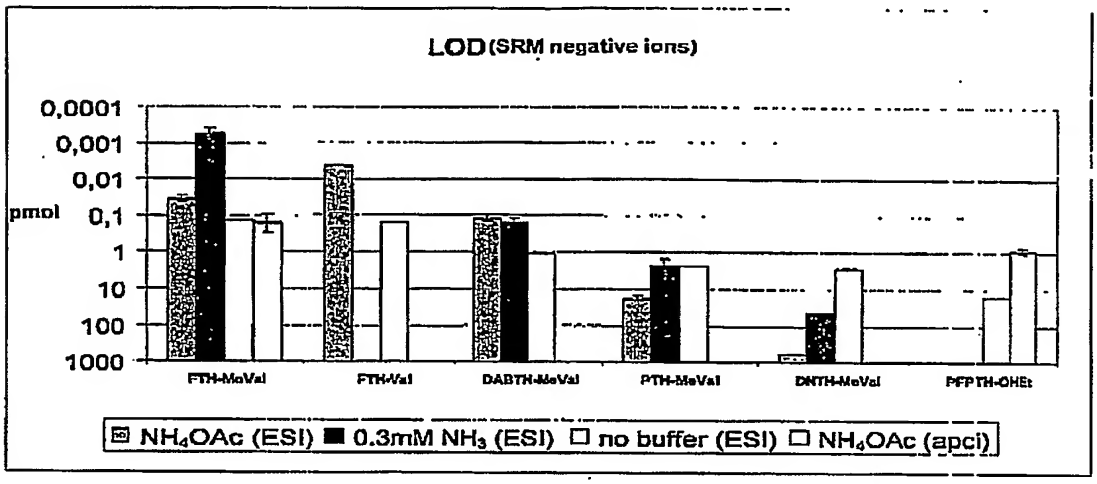


Figure 8. Separation between FTH-Val (9) and FTH-MeVal (10) on Capillary electrophoresis measured with diode array detection. FTH-Val (9) elutes at 7.44 min and FTH MeVal (10) elutes at 8.91 min (phosphate buffer 17 mM, pH 7, 20 mM SDS). 30 kV, 52 cm capillary, 1 nl injected.

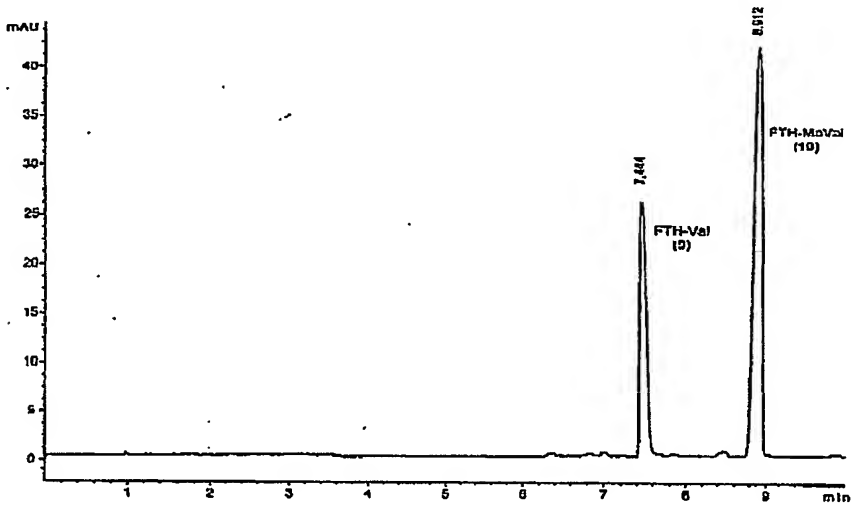
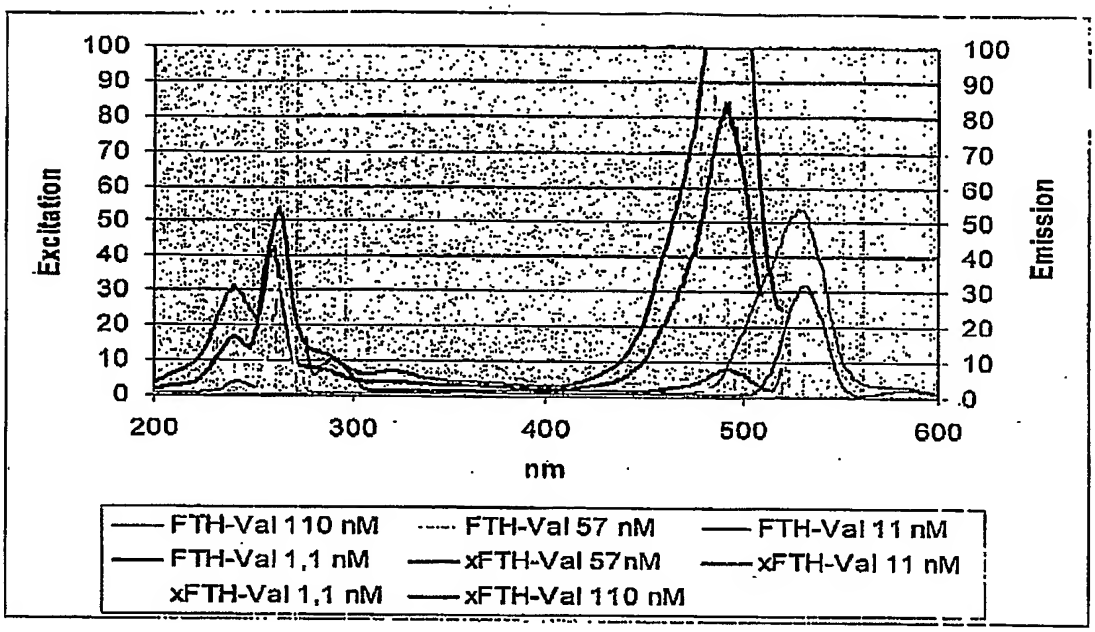
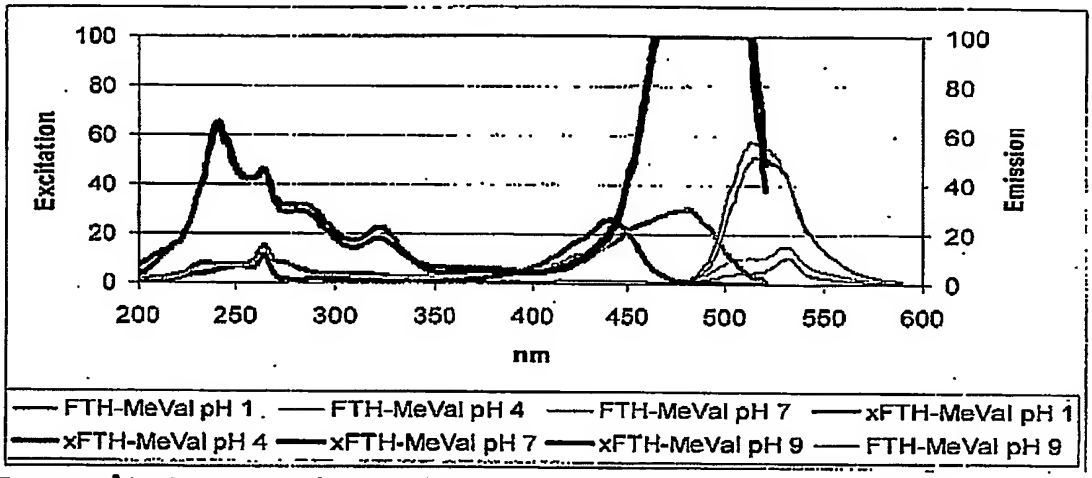


Figure 9. Fluorescens measurements[a]; the excitation and emission spectra of FTH-Val (9) at given concentrations.



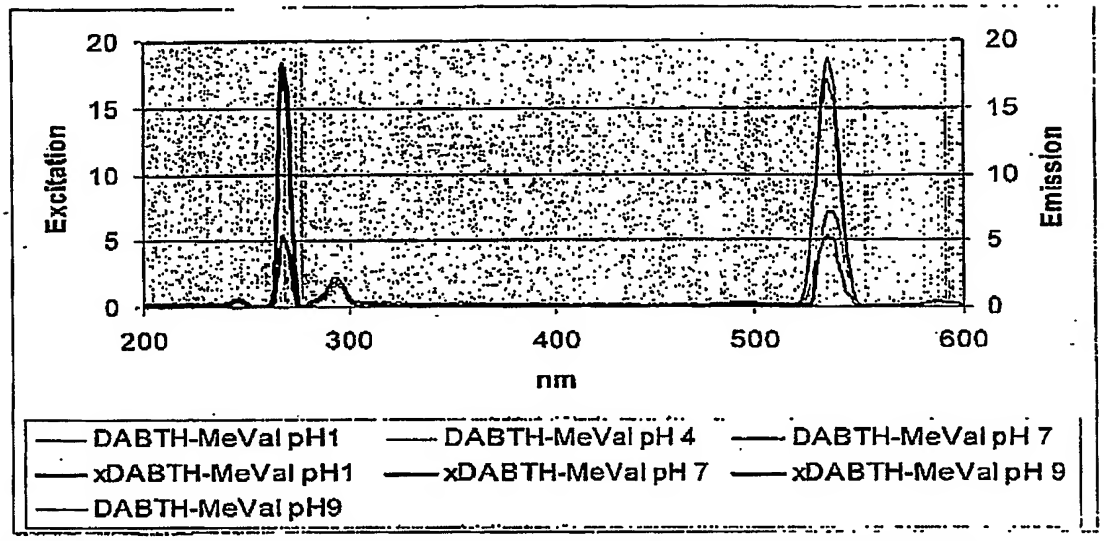
Footnote: [a] FTH-Val (9) solved in acetonitrile:aqueous buffer (1:9), pH 7, different concentrations. Excitation wavelength 265nm, emission scan 275-600nm and emission 517 nm, excitation scan 200-510nm. The bold lines (xFTH-Val) presents the excitation spectra. The thin lines (FTH-Val) presents the emission spectra.

Figure 10. Fluorescens measurements[a]; the excitation and emission spectra of FTH-MeVal (**10**) at pH 1, 4, 7 and 9 at 1.1 μM concentration.



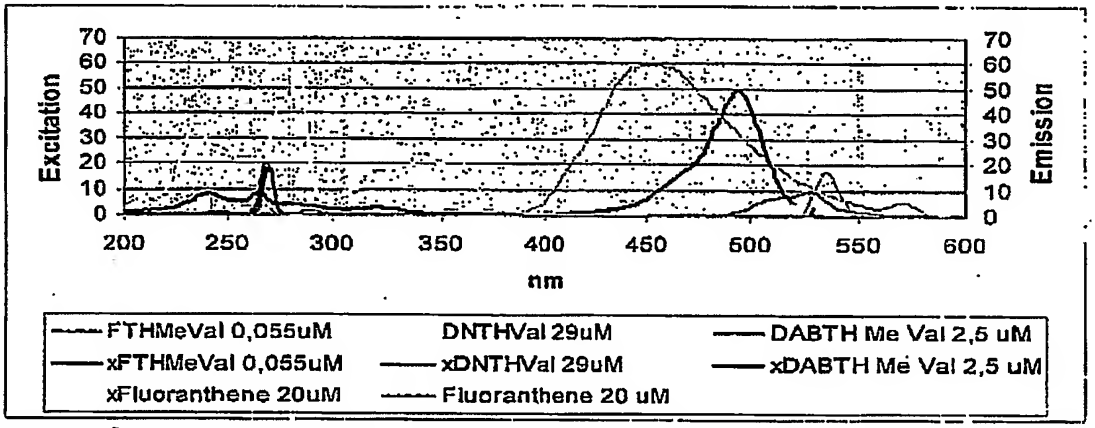
Footnote: [a]Analytes solved in acetonitrile:aqueous buffer (1:9). Slits 5/5, excitation wavelength 265 nm, emission scan 275-600 nm and emission at 530 nm, excitation scan 200-520 nm. The bold lines (xFTH-MeVal pH) presents the excitation spectra. The thin lines (FTH-MeVal pH) presents the emission spectra.

Figure 11. Fluorescens measurements[a]; the excitation and emission spectra of DABTH-MeVal at pH 1, 4, 7 and 9 at 2.5 μM.



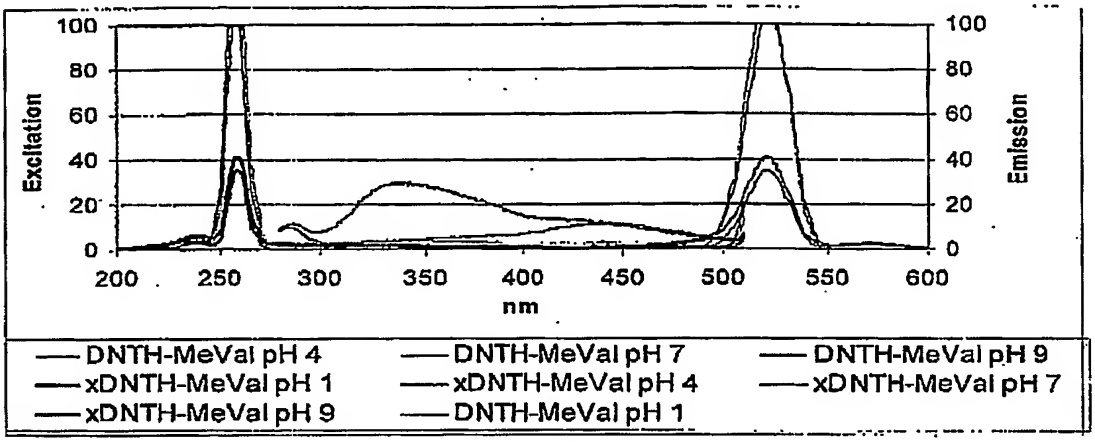
Footnote: [a]Analytes solved in acetonitrile:aqueous buffer (1:9). slits 5/5, excitation wavelength 268 nm, emission scan 280-600 nm and emission 538 nm, excitation scan 200-530 nm. The bold lines (xDABTH-MeVal) presents the excitation spectra. The thin lines (DABTH-MeVal pH) presents the emission spectra.

Figure 12. Comparative studies[a] of excitation and fluorescence emission spectra for the analytes DABTH-MeVal (**6**), DNTH-Val (**7**), FTH-MeVal (**10**) with fluoranten as a reference at the given concentrations



Footnote: [a]Compounds solved in acetonitrile:aqueous buffer (1:9). pH suited for each analyte, slits 5/5 and locked excitation/emission wavelength suited for each analyte. The bold lines represent the excitation spectra. The thin lines represent the emission spectra.

Figure 13. Fluorescens measurements[a]; the excitation and emission spectra of DNTH-MeVal at pH 1, 4, 7 and 9 at 1.5 μM.



Footnote: [a]Solved in acetonitrile:aqueous buffer (1:9). Slits 10/10, excitation wavelength 260 nm, emission scan 270-600 nm and emission 520 nm, excitation scan 200-510 nm. The bold lines (xDNTH-MeVal) represent the excitation spectra. The thin lines (DNTH-MeVal) represent the emission spectra.

Figure 14. UV absorbance for selected analytes in acetonitrile, fluoranthene used as reference.

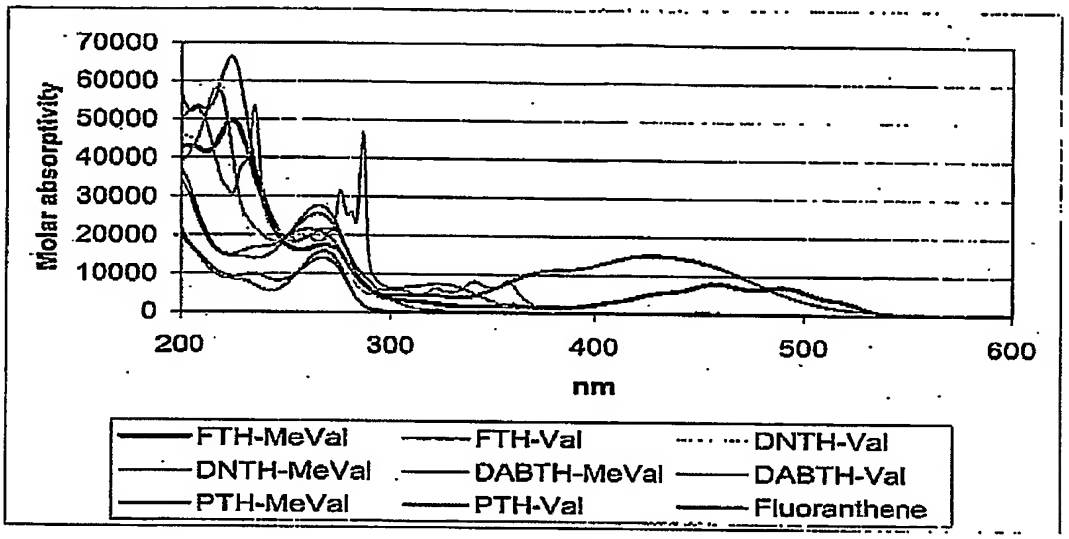


Figure 15. UV absorbance for selected analytes in 0.1 % TFA in acetonitrile/water (1/1), fluoranthene used as reference.

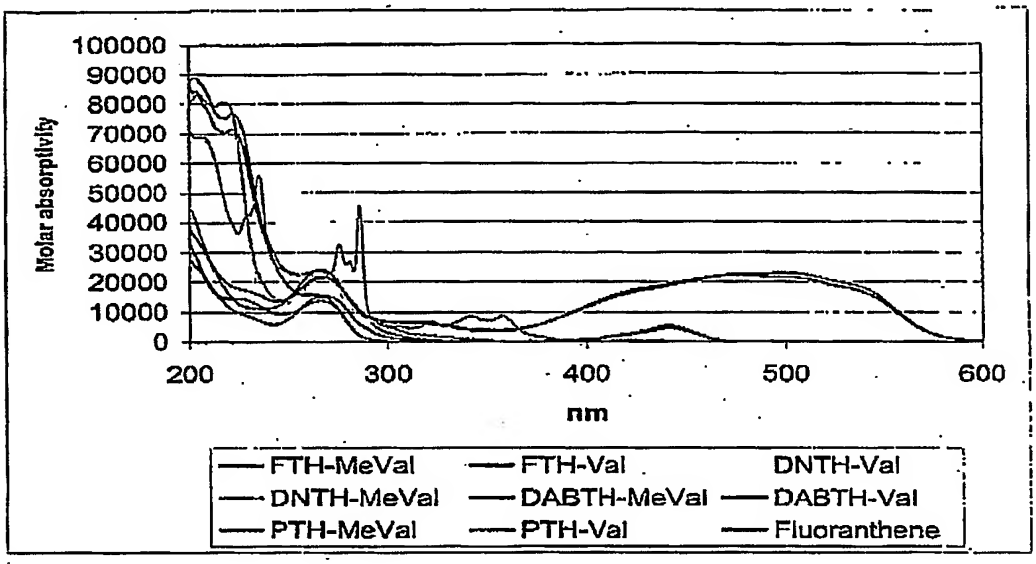


Figure 16. LC-MS/MS analysis of acrylamide adducted human globin by use of the Fluorescent N-*R*-Edman procedure and comparison with reference compounds.

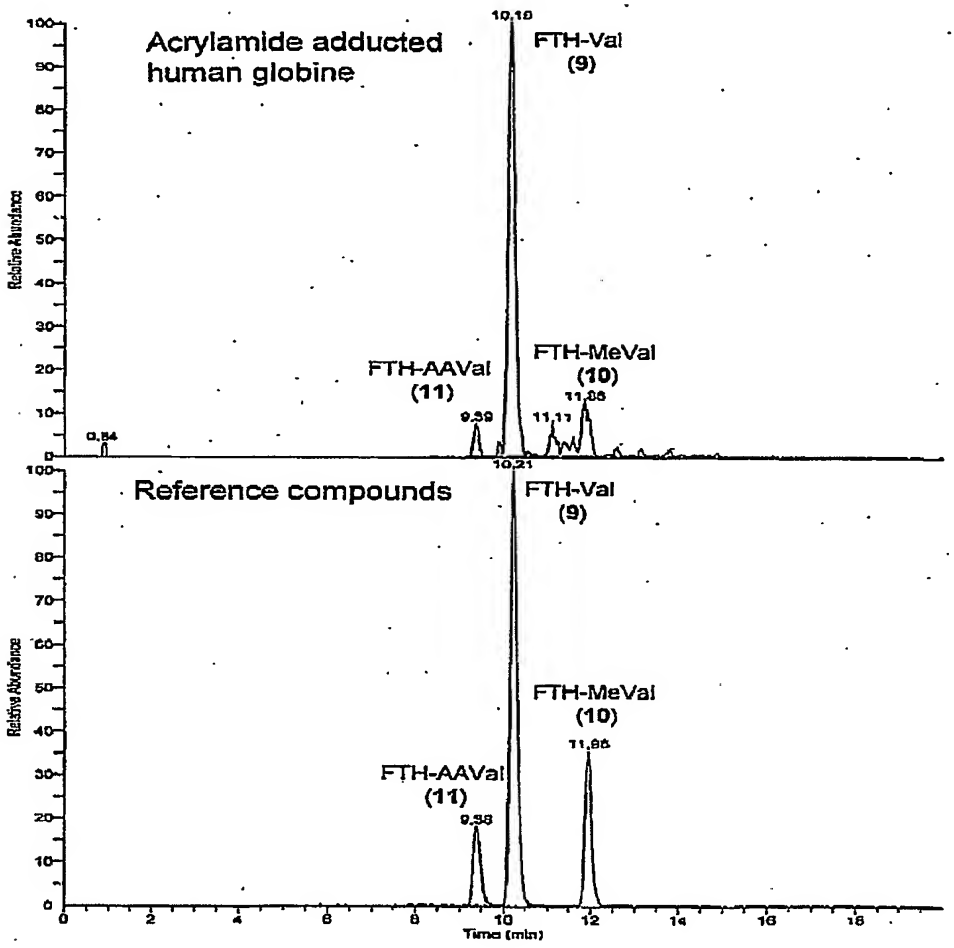
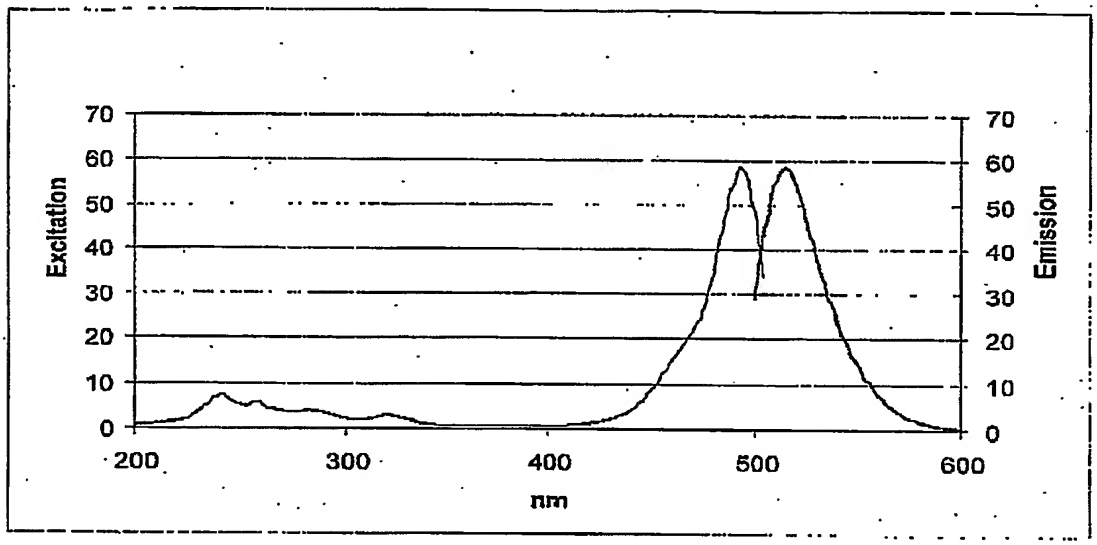


Figure 17. Excitation and emission fluorescence spectra of FTH-MeVal (**10**). The excitation spectrum was recorded at 492 nm and the emission at 515 nm (pH~7; 0.1μM; ACN:H_2O,1:4).



Application Data Sheet

Application Information

Application Type::	Provisional
Subject Matter::	Utility
Suggested Classification::	
Suggested Group Art Unit::	
CD-ROM or CD-R?::	None
Number of CD disks::	
Number of Copies of CDs::	
Sequence Submission?::	None
Computer Readable Form (CRF)::	No
Number of copies of CRF::	0
Title::	METHOD FOR ANALYZING N-TERMINAL PROTEIN ADDUCTS
Attorney Docket Number::	1523-1011
Request for Early Publication?::	No
Request for Non-Publication?::	No
Suggested Drawing Figure::	
Total Drawing Sheets::	17
Small Entity?::	Yes
Latin Name::	
Variety Denomination Name::	
Petition Included?::	No
Petition Type::	
Licensed US Gov't Agency::	
Contract or Grant Numbers::	
Secrecy Order in Parent Appl.?::	No

Applicant Information

Applicant Authority Type:: Inventor
Primary Citizenship Country:: SWEDEN
Status:: Full Capacity
Given Name:: PER
Middle Name::
Family Name:: RYDBERG
Name Suffix::
City of Residence:: HAGERSTEN
State or Province of Residence::
Country of Residence:: SWEDEN
Street of Mailing Address:: TOMTRATTSVAGEN 37
City of Mailing Address:: HAGERSTEN
State or Province of Mailing Address::
Country of Mailing Address:: SWEDEN
Postal or Zip Code of Mailing Address:: SE-129 31

Correspondence Information

Correspondence Customer Number:: 000466

Representative Information

Representative Customer Number::	000466

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::

Foreign Priority Information

Country::	Application Number::	Filing Date::	Priority Claimed::

Assignment Information

Assignee Name::

Street of Mailing Address::

City of Mailing Address::

State or Province of Mailing Address::

Country of Mailing Address::

Postal or Zip Code of Mailing Address::